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(54) Title: HEPATITIS C VIRUS REPLICONS AND REPLICON ENHANCED CELLS

(57) Abstract: The present invention features nucleic acid containing one or more adaptive mutations, and HCV replicon enhanced cells. Adaptive mutations are mutations that enhance HCV replicon activity. HCV replicon enhanced cells are cells having an increased ability to maintain an HCV replicon.

TITLE OF THE INVENTION**HEPATITIS C VIRUS REPLICONS AND REPLICON ENHANCED CELLS****CROSS-REFERENCE TO RELATED APPLICATIONS**

5 The present application claims priority to U.S. Serial No. 60/263,479, filed January 23, 2001, hereby incorporated by reference herein.

BACKGROUND OF THE INVENTION

10 The references cited in the present application are not admitted to be prior art to the claimed invention.

15 It is estimated that about 3% of the world's population are infected with the Hepatitis C virus (HCV). (Wasley, *et al.*, 2000. *Semin. Liver Dis.* 20, 1-16.) Exposure to HCV results in an overt acute disease in a small percentage of cases, while in most instances the virus establishes a chronic infection causing liver inflammation and slowly progresses into liver failure and cirrhosis. (Iwarson, 1994. *FEMS Microbiol. Rev.* 14, 201-204.) In addition, epidemiological surveys indicate an important role of HCV in the pathogenesis of hepatocellular carcinoma. (Kew, 1994. *FEMS Microbiol. Rev.* 14, 211-220, Alter, 1995. *Blood* 85, 1681-1695.)

20 The HCV genome consists of a single strand RNA of about 9.5 kb in length, encoding a precursor polyprotein of about 3000 amino acids. (Choo, *et al.*, 1989. *Science* 244, 362-364, Choo, *et al.*, 1989. *Science* 244, 359-362, Takamizawa, *et al.*, 1991. *J. Virol.* 65, 1105-1113.) The HCV polyprotein contains the viral proteins in the order: C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B.

25 Individual viral proteins are produced by proteolysis of the HCV polyprotein. Host cell proteases release the putative structural proteins C, E1, E2, and p7, and create the N-terminus of NS2 at amino acid 810. (Mizushima, *et al.*, 1994. *J. Virol.* 68, 2731-2734, Hijikata, *et al.*, 1993. *P.N.A.S. USA* 90, 10773-10777.)

30 The non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B presumably form the virus replication machinery and are released from the polyprotein. A zinc-dependent protease associated with NS2 and the N-terminus of NS3 is responsible for cleavage between NS2 and NS3. (Grakoui, *et al.*, 1993. *J. Virol.* 67, 1385-1395, Hijikata, *et al.*, 1993. *P.N.A.S. USA* 90, 10773-10777.) A distinct serine protease located in the N-terminal domain of NS3 is responsible for proteolytic cleavages at the NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions. (Barthenschlager, *et al.*, 1993. *J. Virol.* 67, 3835-3844, Grakoui, *et al.*,

1993. *Proc. Natl. Acad. Sci. USA* 90, 10583-10587, Tomei, *et al.*, 1993. *J. Virol.* 67, 4017-4026.) NS4A provides a cofactor for NS3 activity. (Failla, *et al.*, *J. Virol.* 1994. 68, 3753-3760, De Francesco, *et al.*, U.S. Patent No. 5,739,002.) NS5A is a highly phosphorylated protein concurring interferon resistance. (De Francesco, *et al.*, 2000. 5 *Semin Liver Dis.*, 20(1), 69-83, Pawlotsky, 1999. *J. Viral Hepat. Suppl. 1*, 47-48.) NS5B provides an RNA polymerase. (De Francesco, *et al.*, International Publication Number WO 96/37619, Behrens, *et al.*, 1996. *EMBO* 15, 12-22, Lohmann, *et al.*, 1998. *Virology* 249, 108-118.)

10 Lohmann, *et al.*, *Science* 285, 110-113, 1999, illustrates the ability of a bicistronic HCV replicon to replicate in a hepatoma cell line. The bicistronic HCV replicon contained a neomycin cistron and an NS2-NS5B or an NS3-NS5B cistron. “NS2-NS5B” refers to a NS2-NS3-NS4A-NS4B-NS5A-NS5B polyprotein. “NS3-NS5B” refers to a NS3-NS4A-NS4B-NS5A-NS5B polyprotein.

15 Bartenschlager, European Patent Application 1 043 399, published October 11, 2000 (not admitted to be prior art to the claimed invention), describes a cell culture system for autonomous HCV RNA replication and protein expression. Replication and protein expression is indicated to occur in sufficiently large amounts for quantitative determination. European Patent Application 1 043 399 indicates that prior cell lines or primary cell cultures infected with HCV do not provide favorable

20 circumstances for detecting HCV replication.

SUMMARY OF THE INVENTION

The present invention features nucleic acid containing one or more adaptive mutations, and HCV replicon enhanced cells. Adaptive mutations are 25 mutations that enhance HCV replicon activity. HCV replicon enhanced cells are cells having an increased ability to maintain an HCV replicon.

An HCV replicon is an RNA molecule able to autonomously replicate in a cultured cell and produce detectable levels of one or more HCV proteins. The basic subunit of an HCV replicon encodes for a HCV NS3-NS5B polyprotein along 30 with a suitable 5' UTR-partial core (PC) region and 3' UTR. The 5' UTR-PC region is made up of a 5'UTR region and about 36 nucleotides of the beginning of the core. Additional regions may be present including those coding for HCV proteins or 35 elements such as the complete core, E1, E2, p7 or NS2; and those coding for other types of proteins or elements such as a encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), a reporter protein or a selection protein.

The present application identifies different adaptive mutations that enhance HCV replicon activity. Enhancing replicon activity brings about at least one of the following: an increase in replicon maintenance in a cell, an increase in replicon replication, and an increase in replicon protein expression.

5 Adaptive mutations are described herein by identifying the location of the adaptive mutation with respect to a reference sequence present in a particular region. Based on the provided reference sequence, the same adaptive mutation can be produced in corresponding locations of equivalent regions having an amino acid sequence different than the reference sequence. Equivalent regions have the same
10 function or encode for a polypeptide having the same function.

Replicon enhanced cells are a preferred host for the insertion and expression of an HCV replicon. Replicon enhanced cells are initially produced by creating a cell containing a HCV replicon and then curing the cell of the replicon. The term "replicon enhanced cell" includes cells cured of HCV replicons and progeny
15 of such cells.

Thus, a first aspect of the present invention describes a nucleic acid molecule comprising at least one of the following regions: an altered NS3 encoding region, an altered NS5A encoding region, and an altered EMCV IRES region. The altered region contains one or more adaptive mutations. Reference to the presence of
20 particular adaptive mutation(s) does not exclude other mutations or adaptive mutations from being present. Adaptive mutations are described with reference to either an encoded amino acid sequence or a nucleic acid sequence.

A nucleic acid molecule can be single-stranded or part of a double strand, and can be RNA or DNA. Depending upon the structure of the nucleic acid
25 molecule, the molecule may be used as a replicon or in the production of a replicon. For example, single-stranded RNA having the proper regions can be a replicon, while double-stranded DNA that includes the complement of a sequence coding for a replicon or replicon intermediate may useful in the production of the replicon or replicon intermediate.

30 Preferred nucleic acid molecules are those containing region(s) from SEQ. ID. NOS. 1, 2, or 3, or the RNA version thereof, with one or more adaptive mutations. Reference to "the RNA version thereof" indicates a ribose backbone and the presence of uracil instead of thymine.

35 The presence of a region containing an adaptive mutation indicates that at least one such region is present. In different embodiments, for example, adaptive

mutations described herein are present at least in the NS3 region, in the NS5A region, in the NS3 and NS5A regions, in the EMCV IRES and NS3 regions, in the EMCV and NS5A regions, and in the EMCV IRES, NS3 and NS5A regions.

Another aspect of the present invention describes an expression vector

5 comprising a nucleotide sequence of an HCV replicon or replicon intermediate coupled to an exogenous promoter. Reference to a nucleotide sequence "coupled to an exogenous promoter" indicates the presence and positioning of an RNA promoter such that it can mediate transcription of the nucleotide sequence and that the promoter is not naturally associated with the nucleotide sequence being transcribed. The

10 expression vector can be used to produce RNA replicons.

Another aspect of the present invention describes a recombinant human hepatoma cell. Reference to a recombinant cell includes an initially produced cell and progeny thereof.

Another aspect of the present invention describes a method of making

15 a HCV replicon enhanced cell. The method involves the steps of: (a) introducing and maintaining an HCV replicon into a cell and (b) curing the cell of the HCV replicon.

Another aspect of the present invention describes an HCV replicon enhanced cell made by a process comprising the steps of: (a) introducing and maintaining an HCV replicon into a cell and (b) curing the cell of the HCV replicon.

20 Another aspect of the present invention describes a method of making a HCV replicon enhanced cell comprising an HCV replicon. The method involves (a) introducing and maintaining a first HCV replicon into a cell, (b) curing the cell of the replicon, and (c) introducing and maintaining a second replicon into the cured cell, where the second replicon may be the same or different as the first replicon.

25 Another aspect of the present invention describes an HCV replicon enhanced cell containing a HCV replicon made by the process involving the step of introducing an HCV replicon into an HCV replicon enhanced cell. The HCV replicon introduced into the HCV replicon enhanced cell may be the same or different than the HCV replicon used to produce the HCV replicon enhanced cell. In a preferred

30 embodiment, the HCV replicon introduced into an HCV replicon enhanced cell is the same replicon as was used to produce the enhanced cell.

Another aspect of the present invention describes a method of measuring the ability of a compound to affect HCV activity using an HCV replicon comprising an adaptive mutation described herein. The method involves providing a

35 compound to a cell comprising the HCV replicon and measuring the ability of the

compound to affect one or more replicon activities as a measure of the effect on HCV activity.

Another aspect of the present invention describes a method of measuring the ability of a compound to affect HCV activity using an HCV replicon

5 enhanced cell that comprises an HCV replicon. The method involves providing a compound to the cell and measuring the ability of the compound to effect one or more replicon activities as a measure of the effect on HCV activity.

Other features and advantages of the present invention are apparent from the additional descriptions provided herein including the different examples.

10 The provided examples illustrate different components and methodology useful in practicing the present invention. The examples do not limit the claimed invention. Based on the present disclosure the skilled artisan can identify and employ other components and methodology useful for practicing the present invention.

15 BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1G illustrate the nucleic acid sequence for the pHCVNeo.17 coding strand (SEQ. ID. NO. 3). The different regions of pHCVNeo.17 are provided as follows:

1-341: HCV 5' non-translated region, drives translation of the core-neo fusion protein;

20 342-1181: Core-neo fusion protein, selectable marker;

1190-1800: Internal ribosome entry site of the encephalomyocarditis virus, drives translation of the HCV NS region;

1801-7755: HCV polyprotein from non-structural protein 3 to non-structural protein 5B;

25 1801-3696: Non-structural protein 3 (NS3), HCV NS3 protease/helicase;

3697-3858: Non-structural protein 4A (NS4A), NS3 protease cofactor;

3859-4641: Non-structural protein 4B (NS4B);

4642-5982: Non-structural protein 5A (NS5A);

5983-7755: Non-structural protein 5B (NS5B); RNA-dependent RNA polymerase

30 7759-7989: HCV 3' non-translated region; and

7990-10690 plasmid sequences comprising origin of replication, beta lactamase coding sequence, and T7 promoter.

DETAILED DESCRIPTION OF THE INVENTION

HCV replicons and HCV replicon enhanced cells can be used to produce a cell culture providing detectable levels of HCV RNA and HCV protein. HCV replicons and HCV replicon enhanced hosts can both be obtained by selecting 5 for the ability to maintain an HCV replicon in a cell. As illustrated in the examples provided below, adaptive mutations present in HCV replicons and host cells can both assist replicon maintenance in a cell.

The detectable replication and expression of HCV RNA in a cell culture system has a variety of different uses including being used to study HCV 10 replication and expression, to study HCV and host cell interactions, to produce HCV RNA, to produce HCV proteins, and to provide a system for measuring the ability of a compound to modulate one or more HCV activities.

Preferred cells for use with a HCV replicon are Huh-7 cells and Huh-7 derived cells. "Huh-7 derived cells" are cell produced starting with Huh-7 cells and 15 introducing one or more phenotypic and/or genotypic modifications.

Adaptive Mutations

Adaptive mutations enhance the ability of an HCV replicon to be maintained and expressed in a host cell. Adaptive mutations can be initially selected 20 for using a wild type HCV RNA construct or a mutated HCV replicon. Initial selection involves providing HCV replicons to cells and identifying clones containing a replicon.

Nucleic acid sequences of identified HCV replicons can be determined using standard sequencing techniques. Comparing the sequence of input HCV 25 constructs and selected constructs provides the location of mutations. The effect of particular mutation(s) can be measured by, for example, producing a construct to contain particular mutation(s) and measuring the effect of these mutation(s). Suitable control constructs for comparison purposes include wild type constructs and constructs previously evaluated.

Adaptive mutations were predominantly found in the HCV NS3 and 30 NS5A regions. With the exception of two silent mutations in NS5A and NS5B, consensus mutations occurring in the NS region resulted in changes to the deduced amino acid sequence. Noticeably, the amino acid changes occurred in residues that are conserved in all or a large number of natural HCV isolates. HCV sequences are 35 well known in the art and can be found, for example, in GenBank.

Adaptive mutations described herein can be identified with respect to a reference sequence. The reference sequence provides the location of the adaptive mutation in, for example, the NS3 or NS5A RNA, cDNA, or amino acid sequence. The remainder of the sequence encodes for a functional protein that may have the same, or a different, sequence than the reference sequence.

5 Preferred NS3 and NS5A adaptive mutations and examples of changes that can be made to produce such mutations are shown in Tables 1 and 2. The amino acid numbering shown in Tables 1 and 2 is with respect to SEQ. ID. NO. 1. The nucleotide numbering shown in Tables 1 and 2 is with respect to SEQ. ID. NO. 2.

10 SEQ. ID. NO. 1 provides the amino acid sequence of the Con1 HCV isolate (Accession Number AJ238799). SEQ. ID. NO. 2 provides the nucleic acid sequence of the Con1 HCV isolate.

TABLE 1

15

Preferred NS3 Adaptive Mutations	
Amino Acid	Nucleotide
gly1095ala	G3625C
glu1202gly	A3946G
ala1347thr	G4380A

TABLE 2

Preferred NS5A Adaptive Mutations	
Amino Acid	Nucleotide
Lys@2039	AAA@6458
asn2041thr	A6463C
ser2173phe	C6859T
ser2197phe	C6931T
leu2198ser	T6934C
ala2199thr	G6936A
ser2204arg	C6953A (or G)

"@" refers to an addition.

Preferred adaptive mutations identified with respect to a reference sequence can be produced changing the encoding region of SEQ. ID. NO. 1, or an equivalent sequence, to result in the indicated change. Preferred adaptive mutations 5 provided in Tables 1 and 2 occur in amino acids conserved among different HCV isolates.

Adaptive mutations have different effects. Some mutations alone, or in combination with other mutations, enhance HCV replicon activity. In some cases, two or more mutations led to synergistic effects and in one case, a slightly 10 antagonistic effect was observed.

An adaptive mutation once identified can be introduced into a starting construct using standard genetic techniques. Examples of such techniques are provided by Ausubel, *Current Protocols in Molecular Biology*, John Wiley, 1987-1998, and Sambrook, *et al.*, *Molecular Cloning, A Laboratory Manual*, 2nd Edition, 15 Cold Spring Harbor Laboratory Press, 1989.

HCV replicons containing adaptive mutations can be built around an NS3 region or NS5A region containing one or more adaptive mutations described herein. The final replicon will contain replicon components needed for replication and may contain additional components.

20 SEQ. ID. NO. 2 can be used as a reference point for different HCV regions as follows:

5' UTR- nucleotides 1-341;
Core- nucleotides 342-914;
E1- nucleotides 915-1490;
25 E2- nucleotides 1491-2579;
P7- nucleotides 2580-2768;
NS2- nucleotides 2769-3419;
NS3- nucleotides 3420-5312;
NS4A- nucleotides 5313-5474;
30 NS4B- nucleotides 5475-6257;
NS5A- nucleotides 6258-7598;
NS5B- nucleotides 7599-9371; and
3' UTR- nucleotides 9374-9605.

35 The amino acid sequences of the different structural and non-structural regions is provided by SEQ. ID. NO. 1.

Nucleic acid sequences encoding for a particular amino acid can be produced taking into account the degeneracy of the genetic code. The degeneracy of the genetic code arises because almost all amino acids are encoded for by different combinations of nucleotide triplets or "codons". The translation of a particular codon

5 into a particular amino acid is well known in the art (see, e.g., Lewin *GENES IV*, p. 119, Oxford University Press, 1990). Amino acids are encoded for by RNA codons as follows:

A=Ala=Alanine: codons GCA, GCC, GCG, GCU

C=Cys=Cysteine: codons UGC, UGU

10 D=Asp=Aspartic acid: codons GAC, GAU

E=Glu=Glutamic acid: codons GAA, GAG

F=Phe=Phenylalanine: codons UUC, UUU

G=Gly=Glycine: codons GGA, GGC, GGG, GGU

H=His=Histidine: codons CAC, CAU

15 I=Ile=Isoleucine: codons AUA, AUC, AUU

K=Lys=Lysine: codons AAA, AAG

L=Leu=Leucine: codons UUA, UUG, CUA, CUC, CUG, CUU

M=Met=Methionine: codon AUG

N=Asn=Asparagine: codons AAC, AAU

20 P=Pro=Proline: codons CCA, CCC, CCG, CCU

Q=Gln=Glutamine: codons CAA, CAG

R=Arg=Arginine: codons AGA, AGG, CGA, CGC, CGG, CGU

S=Ser=Serine: codons AGC, AGU, UCA, UCC, UCG, UCU

T=Thr=Threonine: codons ACA, ACC, ACG, ACU

25 V=Val=Valine: codons GUA, GUC, GUG, GUU

W=Trp=Tryptophan: codon UGG

Y=Tyr=Tyrosine: codons UAC, UAU.

Constructs, including subgenomic and genomic replicons, containing one or more of the adaptive mutations described herein can also contain additional mutations. The additional mutations may be adaptive mutations and mutations not substantially inhibiting replicon activity. Mutations not substantially inhibiting replicon activity provide for a replicon that can be introduced into a cell and have detectable activity.

HCV Replicon

HCV replicons include the full length HCV genome and subgenomic constructs. A basic HCV replicon is a subgenomic construct containing an HCV 5' UTR-PC region, an HCV NS3-NS5B polyprotein encoding region, and a HCV 3' UTR. Other nucleic acid regions can be present such as those providing for HCV NS2, structural HCV protein(s) and non-HCV sequences.

The HCV 5' UTR-PC region provides an internal ribosome entry site (IRES) for protein translation and elements needed for replication. The HCV 5' UTR-PC region includes naturally occurring HCV 5' UTR extending about 36 nucleotides into a HCV core encoding region, and functional derivatives thereof. The 5'-UTR-PC region can be present in different locations such as site downstream from a sequence encoding a selection protein, a reporter, protein, or an HCV polyprotein.

Functional derivatives of the 5'-UTR-PC region able to initiate translation and assist replication can be designed taking into structural requirements for HCV translation initiation. (See, for example, Honda, *et al.*, 1996. *Virology* 222, 31-42). The affect of different modifications to a 5' UTR-PC region can be determined using techniques that measure replicon activity.

In addition to the HCV 5' UTR-PC region, non-HCV IRES elements can also be present in the replicon. The non-HCV IRES elements can be present in different locations including immediately upstream the region encoding for an HCV polyprotein. Examples of non-HCV IRES elements that can be used are the EMCV IRES, poliovirus IRES, and bovine viral diarrhea virus IRES.

The HCV 3' UTR assists HCV replication. HCV 3' UTR includes naturally occurring HCV 3' UTR and functional derivatives thereof. Naturally occurring 3' UTR's include a poly U tract and an additional region of about 100 nucleotides. (Tanaka, *et al.*, 1996. *J. Virol.* 70, 3307-3312, Kolykhalov, *et al.*, 1996. *J. Virol.* 70, 3363-3371.) At least *in vivo*, the 3' UTR appears to be essential for replication. (Kolykhalov, *et al.*, 2000. *J. Virol.* 2000 4, 2046-2051.) Examples of naturally occurring 3' UTR derivatives are described by Bartenschlager International Publication Number EP 1 043 399.

The NS3-NS5B polyprotein encoding region provides for a polyprotein that can be processed in a cell into different proteins. Suitable NS3-NS5B polyprotein sequences that may be part of a replicon include those present in different HCV strains and functional equivalents thereof resulting in the processing of NS3-NS5B to

a produce functional replication machinery. Proper processing can be measured for by assaying, for example, NS5B RNA dependent RNA polymerase.

The ability of an NS5B protein to provide RNA polymerase activity can be measured using techniques well known in the art. (See, for example, De

5 Franscesco, *et al.*, International Publication Number WO 96/37619, Behrens, *et al.*, 1996. *EMBO* 15:12-22, Lohmann, *et al.*, 1998. *Virology* 249:108-118.) Preferably, the sequence of the active NS5B is substantially similar as that provided in SEQ. ID. NO. 1, or a wild type NS5B such as strains HCV-1, HCV-2, HCV-BK, HCV-J, HCV-N, HCV-H. A substantially similar sequence provides detectable HCV polymerase

10 activity and contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 amino acid alterations to that present in a HCV NS5B polymerase. Preferably, no more than 1, 2, 3, 4 or 5 alterations are present.

Alterations to an amino acid sequence provide for substitution(s), insertion(s), deletion(s) or a combination thereof. Sites of different alterations can be 15 designed taking into account the amino acid sequences of different NS5B polymerases to identify conserved and variable amino acid, and can be empirically determined.

HCV replicons can be produced in a wide variety of different cells and *in vitro*. Suitable cells allow for the transcription of a nucleic acid encoding for an HCV replicon.

20

Additional Sequences

An HCV replicon may contain non-HCV sequences in addition to HCV sequences. The additional sequences should not prevent replication and expression, and preferably serve a useful function. Sequences that can be used to 25 serve a useful function include a selection sequence, a reporter sequence, transcription elements and translation elements.

Selection Sequence

A selection sequence in an HCV replicon facilitates the identification 30 of a cell containing the replicon. Selection sequences are typically used in conjunction with some selective pressure that inhibits growth of cells not containing the selection sequence. Examples of selection sequences include sequences encoding for antibiotic resistance and ribozymes.

Antibiotic resistance can be used in conjunction with an antibiotic to 35 select for cells containing replicons. Examples of selection sequences providing for

antibiotic resistance are sequences encoding resistance to neomycin, hygromycin, puromycin, or zeocin.

5 A ribozyme serving as a selection sequence can be used in conjunction with an inhibitory nucleic acid molecule that prevents cellular growth. The ribozyme recognizes and cleaves the inhibitory nucleic acid.

Reporter Sequence

10 A reporter sequence can be used to detect replicon replication or protein expression. Preferred reporter proteins are enzymatic proteins whose presence can be detected by measuring product produced by the protein. Examples of reporter proteins include, luciferase, beta-lactamase, secretory alkaline phosphatase, beta-glucuronidase, green fluorescent protein and its derivatives. In addition, a reporter nucleic acid sequence can be used to provide a reference sequence that can be targeted by a complementary nucleic acid. Hybridization of the complementary nucleic acid to 15 its target can be determined using standard techniques.

Additional Sequence Configuration

20 Additional non-HCV sequences are preferable 5' or 3' of an HCV replicon genome or subgenomic genome region. However, the additional sequences can be located within an HCV genome as long as the sequences do not prevent detectable replicon activity. If desired, additional sequences can be separated from the replicon by using a ribozyme recognition sequence in conjunction with a 5' ribozyme.

25 Additional sequences can be part of the same cistron as the HCV polyprotein or can be a separate cistron. If part of the same cistron, the selection or reporter sequence coding for a protein should result in a product that is either active as a chimeric protein or is cleaved inside a cell so it is separated from HCV protein.

30 Selection and reporter sequences encoding for a protein when present as a separate cistron should be associated with elements needed for translation. Such elements include a 5' IRES.

Detection Methods

35 Methods for detecting replicon activity include those measuring the production or activity of replicon RNA and encoded for protein. Measuring includes qualitative and quantitative analysis.

Techniques suitable for measuring RNA production include those detecting the presence or activity of RNA. The presence of RNA can be detected using, for example, complementary hybridization probes or quantitative PCR. Techniques for measuring hybridization between complementary nucleic acid and 5 quantitative PCR are well known in the art. (See for example, Ausubel, *Current Protocols in Molecular Biology*, John Wiley, 1987-1998, Sambrook, *et al.*, *Molecular Cloning, A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989, and U.S. Patent No. 5,731,148.)

10 RNA enzymatic activity can be provided to the replicon by using a ribozyme sequence. Ribozyme activity can be measured using techniques detecting the ability of the ribozyme to cleave a target sequence.

15 Techniques for measuring protein production include those detecting the presence or activity of a produced protein. The presence of a particular protein can be determined by, for example, immunological techniques. Protein activity can be measured based on the activity of an HCV protein or a reporter protein sequence.

Techniques for measuring HCV protein activity vary depending upon the protein that is measured. Techniques for measuring the activity of different non-structural proteins such as NS2/3, NS3, and NS5B, are well known in the art. (See, for example, references provided in the Background of the Invention.)

20 Assays measuring replicon activity also include those detecting virion production from a replicon that produces a virion; and those detecting a cytopathic effect from a replicon producing proteins exerting such an effect. Cytopathic effects can be detected by assays suitable to measure cell viability.

25 Assays measuring replicon activity can be used to evaluate the ability of a compound to modulate HCV activities. Such assays can be carried out by providing one or more test compounds to a cell expressing an HCV replicon and measuring the effect of the compound on replicon activity. If a preparation containing more than one compound is found to modulate replicon activity, individual compounds or smaller groups of compounds can be tested to identify replicon active 30 compounds.

Compounds identified as inhibiting HCV activity can be used to produce replicon enhanced cells and may be therapeutic compounds. The ability of a compound to serve as a therapeutic compound can be confirmed using animal models such as a chimpanzee to measure efficacy and toxicity.

Replicon Enhanced Host Cell

Replicon enhanced cells are initially produced by selecting for a cell able to maintain an HCV replicon and then curing the cell of the replicon. Cells produced in this fashion were found to have an increased ability to maintain a replicon 5 upon subsequent HCV replicon transfection.

Initial transfection can be performed using a wild-type replicon or a replicon containing one or more adaptive mutations. If a wild-type replicon is employed, the replicon should contain a selection sequence to facilitate replicon maintenance.

10 Cells can be cured of replicons using different techniques such as those employing replicon inhibitory agent. In addition, replication of HCV replicons is substantially reduced in confluent cells. Thus, it is conceivable to cure cells of replicons by culturing them at a high density.

15 Replicon inhibitory agents inhibit replicon activity or select against a cell containing a replicon. An example of such an agent is IFN- α . Other HCV inhibitory compounds may also be employed. HCV inhibitor compounds are described, for example, in Llinas-Brunet, *et al.*, 2000. *Bioorg Med Chem Lett*. 10(20), 2267-2270.

20 The ability of a cured cell to be a replicon enhanced cell can be measured by introducing a replicon into the cell and determining efficiency of subsequent replicon maintenance and activity.

EXAMPLES

Examples are provided below to further illustrate different features of 25 the present invention. The examples also illustrate useful methodology for practicing the invention. These examples do not limit the claimed invention.

Example 1: Techniques

This example illustrates the techniques employed for producing and 30 analyzing adaptive mutations and replicon enhanced cells.

Manipulation of Nucleic Acids and Construction of Recombinant Plasmids

Manipulation of nucleic acids was done according to standard protocols. (Sambrook, *et al.*, 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. 35 Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) Plasmid DNA was

prepared from ON culture in LB broth using Qiagen 500 columns according to manufacturer instructions.

Plasmids containing desired mutations were constructed by restriction digestion using restriction sites flanking the mutations or by PCR amplification of the 5 area of interest, using synthetic oligonucleotides with the appropriate sequence. Site directed mutagenesis was carried out by inserting the mutations in the PCR primers. PCR amplification was performed using high fidelity thermostable polymerases or mixtures of polymerases containing a proofreading enzyme. (Barnes, *et al.*, 1994. *Proc. Natl. Acad. Sci.* 91, 2216-2220.) All plasmids were verified by restriction 10 mapping and sequencing.

pHCVneo17.wt contains the cDNA for an HCV bicistronic replicon identical to replicon I₃₇₇neo/NS3-3'/wt described by Bartenschlager (SEQ. ID. NO. 3) (Lohmann, *et al.*, 1999. *Science* 285,110-113, EMBL-genbank No. AJ242652). The plasmid comprises the following elements: 5' untranslated region of HCV comprising 15 the HCV-IRES and part of the core (nt1-377); neomycin phosphotransferase coding sequence; and EMCV IRES; HCV coding sequences from NS3 to NS5B; 3' UTR of HCV.

Plasmid pHCVNeo17.GAA is identical to pHCVNeo.17, except that the GAC triplets (nt. 6934-6939 of pHCVNeo17 sequence) coding for the catalytic 20 aspartates of the NS5B polymerase (amino acids 2737 and 2738 of HCV polyprotein) were changed into GCG, coding for alanine.

Plasmid pHCVNeo17.m0 is identical to pHCVNeo17, except that the triplet AGC (nt. 5335-5337 of pHCVNeo17 sequence) coding for the serine of NS5A protein (amino acid 2204 of HCV polyprotein) was changed into AGA, coding for 25 arginine.

Plasmid pHCVNeo17.m1 is identical to pHCVNeo17, except that the triplet AAC (nt. 4846-4848 of pHCVNeo17 sequence) coding for the asparagine of NS5A protein (amino acid 2041 of HCV polyprotein) was changed into ACC, coding for threonine.

30 Plasmid pHCVNeo17.m2 is identical to pHCVNeo17, except that the triplet TCC (nt. 5242-5244 of pHCVNeo17 sequence) coding for the serine of NS5A protein (amino acid 2173 of HCV polyprotein) was changed into TTC, coding for phenylalanine.

Plasmid pHCVNeo17.m3 is identical to pHCVNeo17, except that the triplet TCC (nt. 5314-5316 of pHCVNeo17 sequence) coding for the serine of NS5A protein (amino acid 2197 of HCV polyprotein) was changed into TTC, coding for phenylalanine.

5 Plasmid pHCVNeo17.m4 is identical to pHCVNeo17, except that the triplet TTG (nt. 5317-5319 of pHCVNeo17 sequence) coding for the leucine of NS5A protein (amino acid 2198 of HCV polyprotein) was changed into TCG, coding for serine.

10 Plasmid pHCVNeo17.m5 is identical to pHCVNeo17, except that an extra triplet AAA coding for lysine was inserted after the triplet GTG (nt. 4840-4843 of pHCVNeo17 sequence), coding for valine 2039 of HCV polyprotein.

15 Plasmid pHCVNeo17.m6 is identical to pHCVNeo17, except that the triplets GAA and GCC (nt. 2329-2331 and 2764-2766 of pHCVNeo17 sequence) coding for the glutamic acid and the alanine of NS3 protein (amino acid 1202 and 1347 of HCV polyprotein) were changed respectively into GGA and ACC, coding for glycine and threonine. The triplet TCC (nt. 5242-5244 of pHCVNeo17 sequence) coding for the serine of NS5A protein (amino acid 2173 of HCV polyprotein) was changed into TTC, coding for phenylalanine; an extra adenosine was inserted into the EMCV IRES (after the thymidine 1736 of the replicon sequence).

20 Plasmid pHCVNeo17.m7 is identical to pHCVNeo17, except that the triplet AAC (nt. 4846-4848 of pHCVNeo17 sequence) coding for the asparagine of NS5A protein (amino acid 2041 of HCV polyprotein) was changed into ACC, coding for threonine; the triplet TCC (nt. 5242-5244 of pHCVNeo17 sequence) coding for the serine of NS5A protein (amino acid 2173 of HCV polyprotein) was changed into TTC, coding for phenylalanine.

25 Plasmid pHCVNeo17.m8 is identical to pHCVNeo17, except that the triplet AAC (nt. 4846-4848 of pHCVNeo17 sequence) coding for the asparagine of NS5A protein (amino acid 2041 of HCV polyprotein) was changed into ACC, coding for threonine; the triplet TCC (nt. 5314-5316 of pHCVNeo17 sequence) coding for the serine of NS5A protein (amino acid 2197 of HCV polyprotein) was changed into TTC, coding for phenylalanine.

30 Plasmid pHCVNeo17.m9 is identical to pHCVNeo17, except that the triplet AAC (nt. 4846-4848 of pHCVNeo17 sequence) coding for the asparagine of NS5A protein (amino acid 2041 of HCV polyprotein) was changed into ACC, coding

for threonine; the triplet TTG (nt. 5317-5319 of pHCVNeo17 sequence) coding for the leucine of NS5A protein (amino acid 2198 of HCV polyprotein) was changed into TCG, coding for serine.

Plasmid pHCVNeo17.m10 is identical to pHCVNeo17, except that the

5 triplet GAA (nt. 2329-2331 of pHCVNeo17 sequence) coding for the glutamic acid of NS3 protein (amino acid 1202 of HCV polyprotein) was changed into GGA, coding for glycine; an extra triplet AAA coding for lysine was inserted after the triplet GTG (nt. 4840-4843 of pHCVNeo17 sequence), coding for valine 2039 of HCV polyprotein.

10 Plasmid pHCVNeo17.m11 is identical to pHCVNeo17, except that the triplet TCC (nt. 5314-5316 of pHCVNeo17 sequence) coding for the serine of NS5A protein (amino acid 2197 of HCV polyprotein) was changed into TTC, coding for phenylalanine. The triplet GCC (nt. 5320-5322 of pHCVNeo17 sequence) coding for the alanine of NS5A protein (amino acid 2199 of HCV polyprotein) was changed into

15 ACC coding for threonine.

Plasmid pHCVNeo17.m12 is identical to pHCVNeo17, except that the triplet AAC (nt. 4846-4848 of pHCVNeo17 sequence) coding for the asparagine of NS5A protein (amino acid 2041 of HCV polyprotein) was changed into ACC, coding for threonine; the triplet TCC (nt. 5314-5316 of pHCVNeo17 sequence) coding for

20 the serine of NS5A protein (amino acid 2197 of HCV polyprotein) was changed into TTC, coding for phenylalanine. The triplet GCC (nt. 5320-5322 of pHCVNeo17 sequence) coding for the alanine of NS5A protein (amino acid 2199 of HCV polyprotein) was changed into ACC coding for threonine.

Plasmid pHCVNeo17.m13 has the same mutations as

25 pHCVNeo17.m8, but also an extra adenine inserted into the EMCV IRES (after the thymidine 1736 of the replicon sequence).

Plasmid pHCVNeo17.m14 has the same mutations as pHCVNeo17.m11, but also an extra adenine inserted into the EMCV IRES (after the thymidine 1736 of the replicon sequence).

30 Plasmid pHCVNeo17.m15 is identical to pHCVNeo17, except that the triplet GCC (nt. 5320-5322 of pHCVNeo17 sequence) coding for the alanine of NS5A protein (amino acid 2199 of HCV polyprotein) was changed into ACC coding for threonine.

Plasmid pRBSEAP.5 is a pHCVNeo.17 derivative where the Neo coding sequence has been replaced with the sequence coding for the human placental alkaline phosphatase corresponding to nucleotides 90-1580 of pBC12/RSV/SEAP plasmid. (Berger, *et al.*, 1988. *Gene* 66, 1-10.)

5

RNA Transfection

Transfection was performed using Huh-7 cells. The cells were grown in Dulbecco's modified minimal essential medium (DMEM, Gibco, BRL) supplemented with 10% FCS. For routine work, cells were passed 1 to 5 twice a 10 week using 1x trypsin/EDTA (Gibco, BRL).

Plasmids were digested with the ScaI endonuclease (New England Biolabs) and transcribed *in vitro* with the T7 Megascript kit (Ambion). Transcription mixtures were treated with DNase I (0.1 U/ml) for 30 minutes at 37°C to completely remove template DNA, extracted according to the procedure of Chomczynski 15 (Chomczynski, *et al.*, 1987. *Anal. Biochem.* 162, 156-159), and resuspended with RNase-free phosphate buffered saline (rfPBS, Sambrook, *et al.*, 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

RNA transfection was performed as described by Liljestrom, *et al.*, 20 1991. *J. Virol.* 6, 4107-4113, with minor modifications. Subconfluent, actively growing cells were detached from the tissue culture container using trypsin/EDTA. Trypsin was neutralised by addition of 3 to 10 volumes of DMEM/10%FCS and cells were centrifuged for 5 minutes at 1200 rpm in a Haereus table top centrifuge at 4°C. Cells were resuspended with ice cold rfPBS by gentle pipetting, counted with a 25 haemocitometer, and centrifuged as above. rfPBS wash was repeated once and cells were resuspended at a concentration of 1-2 x 10⁷ cell/ml in rfPBS. Aliquots of cell suspension were mixed with RNA in sterile eppendorf tubes. The RNA/cell mixture was immediately transferred into the electroporation cuvette (precooled on ice) and pulsed twice with a gene pulser apparatus equipped with pulse controller (Biorad). 30 Depending on the experiment, 0.1, 0.2 or 0.4 cm electrode gap cuvettes were used, and settings adjusted (Table 3).

TABLE 3

Cuvette	Volume	Voltage	Capacitance	Resistance	RNA
gap (cm)	(μ l)	(Volts)	(μ Fa)	(ohm)	(μ g)
0.1	70	200	25	infinite	1-10
0.2	200	400	25	infinite	5-20
0.4	800	800	25	infinite	15-100

After the electric shock, cells were left at room temperature for 1-10 minutes (essentially the time required to electroporate all samples) and subsequently diluted with at least 20 volumes of DMEM/10%FCS and plated as required for the experiment. Survival and transfection efficiency were monitored by measuring the neutral red uptake of cell cultured for various days in the absence or in the presence of neomycin sulfate (G418). With these parameters, survival of Huh-7 cells was usually 40-60% and transfection efficiency ranged between 40% and 100%.

Sequence Analysis of Replicon RNAs

The entire NS region was recloned from 3 different transfection experiments performed with HCVNeo.17 RNA. RNA was extracted from selected clones either using the Qiagen RNAeasy minikit following manufacturer instructions or as described by Chomczynski, *et al.*, 1987. *Anal. Biochem.* 162, 156-159.

Replicon RNAs (5 μ g of total cellular RNA) were retro-transcribed using oligonucleotide HCVG34 (5'- ACATGATCTGCAGAGAGGCCAGT-3'; SEQ. ID. No. 4) and the Superscript II reverse transcriptase (Gibco, BRL) according to manufacturer instructions, and subsequently digested with 2 U/ml Ribonuclease H (Gibco BRL). The cDNA regions spanning from the EMCV IRES to the HCV 3' end were amplified by PCR using oligonucleotides HCVG39 (5'- GACASGCTGTGATAWATGTCTCCCC-3'; SEQ. ID. NO. 5) and CITE3 (5'- TGGCTCTCCTCAAGCGTATTG-3'; SEQ. ID. NO. 6) and the LA Taq DNA polymerase (Takara LA Taq).

Amplified cDNAs were digested with the KpnI endonuclease (New England Biolabs) and the 5.8 kb fragments were gel purified and ligated to the 5.6 kb vector fragment (purified from plasmid pRBSEAP.5 digested with KpnI) using T4

DNA ligase (New England Biolabs) according to manufacturer instructions. Ligated DNAs were transformed by electroporation in DH10B or JM119 strains of *E. coli*.

In the case of NS5A region, total RNA isolated from 3 clones, (HB77, HB60 and HB68) was extracted and used for RT-PCR. 5 μ g of total RNA plus 20 pmole of AS61 oligo (5'-ACTCTCTGCAGTCAGCGGCTCA-3', RT antisense oligo; SEQ. ID. NO. 7) were heated 5 minutes at 95°C, then DMSO (5% f.c.), DTT (10 mM f.c.), 1 mM dNTP (1 mM f.c.), 1x Superscript buffer (1 x f.c.), and 10 u Superscript (Gibco) were added to a total volume of 20 μ l and incubated 3 hours at 42°C. 2 μ l of this RT reaction were used to perform PCR with oligos S39 (5'-CAGTGGATGAACCGGCTGATA-3', sense; SEQ. ID. NO. 8) or S41 (5'-GGGGCGACGGCATCATGCAAACC-3', sense; SEQ. ID. NO. 9) and B43 (5'-CAGGACCTGCAGTCTGTCAAAGG-3', antisense; SEQ. ID. NO. 10) using Elongase Enzyme Mix (Gibco) according the instruction provided by the manufacturer. The resulting PCR fragment was cloned in pCR2.1 vector using the TA Cloning kit (Invitrogen) and transformed in Top10F' bacterial strain.

Plasmid DNA was prepared from ON culture of the resulting ampicillin resistant colonies using Qiagen 500 columns according to manufacturer instructions. The presence of the desired DNA insert was ascertained by restriction digestion, and the nucleotide sequence of each plasmid was determined by automated sequencing. Nucleotide sequences and deduced amino acids sequences were aligned using the GCG software.

TaqMan

TaqMan analysis was typically performed using 10 ng of RNA in a reaction mix (TaqMan Gold RT-PCR kit, Perkin Elmer Biosystems) either with HCV specific oligos/probe (oligo 1: 5'-CGGGAGAGCCATAGTGG-3'; SEQ. ID. NO. 11, oligo 2: 5'-AGTACCACAAGGCCTTCG-3'; SEQ. ID. NO. 12, probe: 5'-CTGCGGAACCGGTGAGTACAC-3'; SEQ. ID. NO. 13) or with human GAPDH specific oligos/probe (Pre-Developed TaqMan Assay Reagents, Endogenous Control Human GAPDH, Part Number 4310884E, Perkin Elmer Biosystems). PCR was performed using a Perkin Elmer ABI PRISM 7700 under the following conditions: 30 minutes at 48°C (the RT step), 10 minutes at 95°C and 40 cycles: 15 seconds at 95°C and 1 minute at 60°C. Quantitative calculations were obtained using the Comparative C_T Method (described in User Bulletin #2, ABI PRISM 7700 Sequence Detection System, Applied Biosystem, Dec 1997) considering the level of GAPDH mRNA

constant. All calculations of HCV RNA are expressed as fold difference over a specific control.

Antibodies and Immunological Techniques

5 Mouse monoclonal antibody (anti-NS3 mab10E5/24) were produced by standard techniques. (Galfré and Milstein, 1981. *Methods in Enzymology* 73, 1-46.) Purified recombinant protein was used as an immunogen. (Gallinari, *et al.*, 1999. *Biochemistry* 38, 5620-5632.)

10 For Cell-ELISA analysis, transfected cells were monitored for expression of the NS3 protein by ELISA with the anti-NS3 mab 10E5/24. Cells were seeded into 96 well plates at densities of 40,000, 30,000, 15,000 and 10,000 cells per well and fixed with ice-cold isopropanol at 1, 2, 3 and 4 days post-transfection, respectively. The cells were washed twice with PBS, blocked with 5% non-fat dry milk in PBS + 0.1% Triton X100 + 0.02% SDS (PBSTS) and then incubated

15 overnight at 4°C with 10E5/24 mab diluted 1:2000 in Milk/PBSTS. After washing 5 times with PBSTS, the cells were incubated for 3 hours at room temperature with anti-mouse IgG Fc specific alkaline phosphatase conjugated secondary antibody (Sigma A-7434), diluted 1:2000 in Milk/PBSTS. After washing again as above, the reaction was developed with *p*-nitrophenyl phosphate disodium substrate (Sigma 104-105) and the absorbance at 405 nm read at intervals.

20 The results were normalized by staining with sulforhodamine B (SRB Sigma S 1402) to determine cell numbers. The alkaline phosphatase substrate was removed from the wells and the cells washed with PBS. The plates were then incubated with 0.4% SRB in 1% acetic acid for 30 minutes (200 µl/well), rinsed 4 times in 1% acetic acid, blotted dry and then 200 µl/well of 10mM Tris pH 10.5 added. After mixing, the absorbance at 570 nm was read.

Neutral Red/ Crystal Violet Staining of Foci

25 The survival of transfected cells in the absence or presence of G418 was monitored by staining of foci/clones with neutral red *in vivo* with subsequent crystal violet staining. The medium was removed from the cells and replaced with fresh medium containing 0.0025% neutral red (Sigma N2889) and the cells incubated for 3 hours at 37°C. Cells were washed twice with PBS, fixed in 3.5% formaldehyde for 15 minutes, washed twice again in PBS and then with distilled water and the 30 number of (live) foci counted. The cells could then be re-stained with crystal violet

by incubating with an 0.1% crystal violet (Sigma C0775) solution in 20% methanol for 20 minutes at room temperature, followed by 3 washes in 20% methanol and a wash with distilled water.

5 *Preparation Of Cells Cured Of Endogenous Replicon*

Replicon enhanced cells designated 10IFN and Cl.60/cu were produced using different HCV inhibitory agents. Based on the techniques described herein additional replicon enhanced clones can readily be obtained.

10IFN was obtained by curing a Huh-7 cell of a replicon using human IFN- α 2b. Huh-7 cells containing HCV replicons (designated HBI10, HBIII4, HBIII27 and HBIII18) were cultured for 11 days in the presence of 100 U/ml recombinant human IFN- α 2b (Intron-A, Schering-Plough), and subsequently for 4 days in the absence of IFN- α 2b. At several time points during this period, the clones were analyzed for the presence of HCV proteins and RNA by Western and Northern blotting. After 7 days of incubation with IFN- α 2b, HCV proteins could no longer be detected in any of these clones by Western blotting and similar effects were seen with RNA signals in Northern blots. IFN- α 2b treated cells were stored in liquid nitrogen until used for transfection experiments.

Cl.60/cu was obtained by curing a Huh-7 cell of a replicon using an HCV inhibitory compound. The presence of HCV RNA was determined using PCR (TaqMan) at 4, 9, 12 and 15 days. From day 9 the amount of HCV RNA was below the limit of detection. To further test the disappearance of the replicon, 4 million cells of cured Clone 60 cells (after the 15 days of treatment) were plated in the presence of 1 mg/ml G-418. No viable cells were observed, confirming that absence of HCV replicons able to confer G-418 resistance.

Example 2: Selection and Characterization of Cell Clones Containing Functional HCV Replicons

Huh-7 cells ($2-8 \times 10^6$) were transfected by electroporation with *in vitro* transcribed replicon RNAs (10–20 μ g), plated at a density ranging from 2.5×10^3 to $10 \times 10^3/\text{cm}^2$, and cultured in the presence of 0.8–1 mg/ml G418. The majority of replicon transfected cells became transiently resistant to G418 and duplicated normally for 7 to 12 days in the presence of the drug, while cells transfected with irrelevant RNAs and mock transfected cells did not survive more than 7 days (data not shown). Transient resistance to G418 was likely due to persistence of the Neo protein

expressed from the transfected RNA, since it was observed also with mutated replicons unable to replicate. Approximately 2 weeks after transfection, transient resistance declined, most cells died and small colonies of cells permanently resistant to the antibiotic became visible in samples transfected with HCVNeo.17 RNA, but

5 not in cells transfected with other replicon RNAs.

In several experiments, the frequency of G418 resistant clones ranged between 10 and 100 clones per 10^6 transfected cells. About 20 G418 resistant colonies were isolated, expanded and molecularly characterized. PCR and RT-PCR analysis of nucleic acids indicated that all clones contained HCV RNA but not HCV

10 DNA, demonstrating that G418 resistance was due to the presence of functional replicons (data not shown). This result was confirmed by Northern blot analysis and metabolic labeling with 3 H-uridine, which revealed the presence of both genomic and antigenomic HCV RNAs of the expected size (data not shown). Lastly, western blot, immunoprecipitation and immunofluorescence experiments showed that these clones
15 expressed all HCV non-structural proteins as well as Neo protein (data not shown).

Clones differed in terms of cell morphology and growth rate. Replicon RNA copy number (500-10000 molecules/cell) and viral protein expression also varied between different clones (data not shown). However, the amount of replicon RNA and proteins also varied with passages and with culture conditions and was
20 higher when cells were not allowed to reach confluence, suggesting that replicons replicated more efficiently in dividing cells than in resting cells. Processing of the viral polyprotein occurred with kinetics similar to those observed in transfected cells.

25 Interestingly, in all tested clones HCV replication was efficiently inhibited by treating the cells with IFN- α 2b. The EC₅₀ was between 1 and 10 U/ml, depending on the clone.

Example 3: Identification of Adaptive Mutations

The low number of G418 resistant clones derived from HCVNeo.17 RNA transfection suggested that replication could require mutation(s) capable of
30 adapting the replicon to the host cell (adaptive mutations) and/or that only a small percentage of Huh-7 cells were competent for HCV replication. To verify the first hypothesis, mutations in replicons RNAs derived from selected cell clones were identified.

RNA sequences for different replicons were determined using standard techniques. Such techniques involved isolating RNA from several independent clones, reverse transcription to produce cDNA, amplifying cDNAs by PCR and cloning into an appropriate vector. The cDNA spanning almost the entire HCV NS region (126 bp at the 3' end of the EMCV IRES and 5650 bp of the HCV NS region (*i.e.*, the entire NS ORF and 298 nucleotides at the 3' end) from 5 clones (HBI10, HBIII12, HBIII18, HBIII27, HBIV1) were recloned and sequenced. In addition, the NS5A coding region (nt. 4784-6162) from 3 additional clones (HB 77, HB 68 and HB 60) were recloned and sequenced.

5 To discriminate mutations present in the replicon RNA from those derived from the cloning procedure, at least 2 isolates derived from independent RT-PCR experiments were sequenced for each cell clone. Comparison of the nucleotide sequences with the parental sequence indicated that each isolate contained several mutations (Tables 4A and 4B).

10

15

TABLE 4A

Cell clone	HBIII 12		HBIII 18		HBI 10		HBIII 27		
	isolate	4	29	28	61	12	43	13	72
		1674-7460	1674-7460	1674-7460	1674-7460	1674-7460	1674-7460	1674-7460	
EMCV IRES 126 bp	A @ 1736	A @ 1736		C 1752 T				T 1678 C	
NS3 1895 bp	G 2009 C A 2698 G G 2764 A A 3256 G T 3273 C	A 2330 G C 2505 T G 2764 A T 3085 C	T 2150 C C 2196 A T 3023 A T 3134 C C 3267 T	T 2015 C A 2338 G C 2616 T A 2664 G A 3148 G T 3286 C C 3615 T C 3657 T	T 1811 A A 2330 G T 2666 C T 3395 C	A 2330 G A 2882 G T 3673 C	G 2009 C T 2015 C C 2336 G A 3130 T A 3401 G A 3518 C	G 2009 C C 2052 A G 2644 A C 2803 A T 2823 A T 3692 C	
NS4A 161 bp	T 3790 C		A 3847 G	T 3827 A	T 3742 C		A 3743 G	A 3797 G	
NS4B 782 bp	T 3869 C A 4107 G T 4185 C A 4428 G	C 4283 T C 4429 T	G 4300 A	A 4136 G A 4261 G G 4309 A A 4449 G	T 4290 C	A 4053 G A 2496 C T 4316 G	G 3880 A T 4200 C A 4366 G	C 4547 T	

TABLE 4A

Cell clone	HBIII 12		HBIII 18		HBI 10		HBIII 27	
isolate	4	29	28	61	12	43	13	72
	1674-7460	1674-7460	1674-7460	1674-7460	1674-7460	1674-7460	1674-7460	1674-7460
NSSA 1340 bp	A 4847 C <i>G 5158 A</i>	G 4728 A <i>A 4845 G</i>	C 5243 T <i>A 5486 G</i>	C 4729 A <i>T 4993 C</i>	A 4694 T <i>AAA @ 4842</i>	A 4675 G <i>A 4761 G</i>	A 4855 G <i>C 5006 T</i>	A 4888 G <i>C 4985 T</i>
	G 5175 C	C 5243 T	C 5596 T	G 5095 A	T 5237 C	AAA @ 4842 <i>T 5368 C</i>	T 5318 C	T 5030 A
	C 5243 T	<u>G 5512 T</u>	G 5823 A	T 5334 C		A 5574 G	T 5090 A	
	C 5390 T	<u>A 5521 G</u>		A 5374 T		G 5866 A	T 5318 C	
	A 5719 G	<u>A 5600 G</u>		T 5379 A		A 5328 G		
		A 5740 C		T 5480 C		A 5399 G		
				A 5513 G		A 5574 G		
				T 5977 C				
NS5B 1477 bp	T 6316 C <i>T 6589 C</i> <i>T 7370 C</i>	A 6406 G <i>G 6756 A</i> <i>G 6963 T</i>	T 6074 C <i>A 6541 G</i> <i>A 6732 G</i>	A 6150 G <i>A 6218 G</i> <i>T 7352 A</i>	A 6911 G	A 5986 G <i>T 6099 C</i> <i>C 6141 T</i>	G 6479 C <i>C 6870 T</i> <i>A 7213 G</i>	G 6156 A <i>G 7434 A</i> <i>T 7444 C</i>

Clone name and isolate number are indicated in the first and second row, respectively.
The first and the last nucleotide of the region that was recloned and sequenced are indicated in the third row.

5

Nucleotide (IUB code) substitutions are indicated with the original nucleotide, its position and mutated nucleotide.

Nucleotide(s) insertions are indicated with the nucleotide(s), the symbol @ and the position of the nucleotide preceding insertion.

10

Numbering refers to the first nucleotide of the replicon sequence (EMBL-genbank No. AJ242652).

The region in which mutations are located and the nucleotide length of each region are indicated in the left most column.

Silent mutations are in italic.

Non sense mutations are underlined.

15

Consensus mutations are bold.

TABLE 4B

Cell clone	HBIV1		HB 77		HB 68		HB 60	
isolate	85	93	10	14	42	1	13	7
	1674-7460	1674-7460	4784-6162	4465-6162	4784-6162	4465-6162	4784-6162	4784-6162
EMCV IRES 126 bp		<i>A @ 1736</i>						
NS3 1895 bp	A 3403 G	A 2572 G <i>A 3454 G</i>						

TABLE 4B

Cell clone	HBIV1		HB 77		HB 68		HB 60	
isolate	85	93	10	14	42	1	13	7
	1674-7460	1674-7460	4784-6162	4465-6162	4784-6162	4465-6162	4784-6162	4784-6162
NS4A 161 bp								
NS4B 782 bp	A 4084 G	<u>C 3892 T</u>						
NS5A 1340 bp	T 4742 C C 5315 T <u>G 5431 T</u> T 5751 C T 5797 C	A 4847 C A 5225 G C 5315 T G 5320 A T 5356 A G 5523 A T 5888 A	C 4813 T G 5060 C C 5337 A	A 4699 C A 5161 G C 5337 A A 5459 G T 5977 C	T 5171 G C 5298 T C 5337 A A 5639 G A 5969 G	T 4587 C T 4972 C A 5094 G A 5278 G G 5320 A C 5532 T	A 4821 G G 5320 A A 5414 G T 5601 G C 5808 T	C 5337 G C 5551 T G 5806 A
NS5B 1477 bp	T 6144 A A 6365 G A 6656 G A 6677 G T 6855 C T 6947 A T 6997 C G 7041 T A 7187 C	T 6855 C A 7135 G T 7171 C						

See Table 4A legend.

The frequency of mutations ranged between 1.7×10^{-3} and 4.5×10^{-3}

5 (average 3×10^{-3}). The majority of mutations were nucleotide substitutions, although insertions of 1 or more nucleotides were also observed (Tables 4A and 4B).

10 Approximately 85% of the mutations found only in 1 isolate (non-consensus) were randomly distributed in the recloned fragment, and possibly include mis-incorporation during the PCR amplifications. Conversely, the remaining 15% of the mutations were common to 2 or more isolates derived from independent RT-PCR experiments (consensus mutations), and presumably reflected mutations present in the template RNA.

15 Consensus mutations were found in all isolates and were either common to isolates derived from the same clone (consensus A), or to isolates derived from different clones (consensus B). Analysis of additional isolates derived from the same cell clones indicated that consensus A mutations were not always present in all isolates derived from one clone (data not shown). This observation, together with the

presence of consensus B mutations, suggests that, even within a single cell clone, replicons exist as quasi-species of molecules with different sequences.

At variance with non-consensus mutations, consensus mutations were not randomly distributed but were clustered in the regions coding for the NS5A protein (frequency 1×10^{-3}) and for the NS3 protein (frequency 0.5×10^{-3}). Only one consensus mutation was found in the region coding for the NS5B protein (frequency 0.1×10^{-3} nucleotides) and none in the regions coding for NS4A and NS4B. Interestingly, 1 consensus mutation was observed also in the EMCV IRES.

With the exception of 2 silent mutations found in NS5A and NS5B, consensus mutations occurring in the NS region resulted in changes in the deduced amino acid sequence (Tables 5A and 5B). Noticeably, these amino acid changes occurred in residues that are conserved in all or most natural HCV isolates. Interestingly, clones HB 77 and HB 60 displayed different nucleotide substitutions (C5337A and C5337G, respectively) resulting in the same amino acidic mutation (S 15 2204 R).

TABLE 5A

Cell clone	HBIII 12		HBIII 18		HBI 10		HBIII 27	
isolate	4	29	28	61	12	43	13	72
NS3	G 1095 A A 1347 T	E 1202 G A 1347 T			E 1202 G	E 1202 G	G 1095 A	G 1095 A
NS4A								
NS4B								
NS5A	N 2041 T S 2173 F	S 2173 F	S 2173 F	E 2263	K @ 2039	K @ 2039	L 2198 S R 2283 R	L 2198 S R 2283 R
NS5B								

See Table 4A legend.

TABLE 5B

Cell clone	HBIV1		HB 77		HB 68		HB 60	
isolate	85	93	10	14	42	1	13	7
NS3								
NS4A								
NS4B								
NS5A	S 2197 F S 2197 F A 2199 T	N 2041 T	S 2204 R	S 2204 R	S 2204 R	A 2199 T	A 2199 T	S 2204 R
NS5B	N 2710 N	N 2710 N						

See Table 4A legend.

5 Example 4: Functional Characterization of Consensus Mutations

The identification of consensus mutations in recloned replicons indicated that replication proficiency of replicon RNAs contained in selected cell clones depended from the presence of such mutations. To substantiate this hypothesis, the effect of several consensus mutations on replication were analyzed.

10 Consensus mutations found in the NS5A region were more closely analyzed. Consensus mutations were segregated from the non-consensus ones, and pHCVNeo.17 derivatives containing single or multiple consensus mutations were constructed (Table 6).

TABLE 6

Construct	Consensus mutations			G418 cfu/10 ³ transfected cells
	NS3	NS5A	EMCV IRES	
pHCVNeo17.wt				0-3
pHCVNeo17.GAA				0
pHCVNeo17.m0		S2204R		30-130
pHCVNeo17.m1		N2041T		0-3
pHCVNeo17.m2		S2173F		15-60
pHCVNeo17.m3		S2197F		160-500
pHCVNeo17.m4		L2198S		30-50

TABLE 6

Construct	Consensus mutations			G418 cfu/10 ⁵ transfected cells
pHCVNeo17.m5	NS3 E1202G; A1347T E1202G	NS5A <u>K@2039</u>	EMCV IRES	25-55
pHCVNeo17.m6		S2173F	Extra A	13-100
pHCVNeo17.m7		N2041T; S2173F		0-1
pHCVNeo17.m8		N2041T; S2197F		360-500
pHCVNeo17.m9		N2041T; L2198S		140-170
pHCVNeo17.m10		<u>K@2039</u>		1060
pHCVNeo17.m11		S2197F; A2199T		900
pHCVNeo17.m12		N2041T; S2197F; A2199T		>1000
pHCVNeo17.m13		N2041T; S2197F	Extra A	100
pHCVNeo17.m14		S2197F; A2199T	Extra A	>500
pHCVNeo17.m15		A2199T		300-600

Huh-7 cells (2×10^6) were transfected with 10 μ g of RNA transcribed from the indicated constructs. Approximately 2×10^5 cells were plated in a 10 cm tissue culture dish and cultured with 1 mg/ml G418 for 20 days.

Colonies surviving selection were stained with crystal violet and counted.

5

RNAs transcribed *in vitro* from these constructs were transfected in Huh-7 cells and the affect on replication was estimated by counting neomycin resistant colonies (G418 cfu). As shown in Table 6, all but 1 construct containing single consensus mutations showed a significant increase on G418 cfu efficiency, thus indicating that the corresponding mutations improved replication. Noticeably, 2 mutants containing single mutations in NS5A (m3 and m15) were clearly more effective than all other single mutants. Results of mutants containing 2 or more mutations, indicated the presence of a synergistic effect in some combinations (m8, m9, m11 and possibly m10), but also a slightly antagonistic effect in 1 mutant (m7).

10

Example 5: Replicon Replication in the Absence of Selection

Replication of HCV replicons in the absence of a G418 selection was detected using quantitative PCR (TaqMan). At 24 hours post-transfection a large amount of replicon RNA was detected in cells transfected with all replicons, including the GAA control replicon containing mutations in the catalytic GDD motif of the NS5B polymerase. This result suggested that analysis at very early time points (up to 48 hour post-transfection) essentially measured the input RNA. Northern blot analysis also indicated that after 24 hours the majority of the transfected RNA was degraded intracellularly (data not shown).

Analysis at later time points showed that the amount of replicon RNA was considerably reduced at 4 days and eventually became undetectable (6/8 days) in cells transfected with replicon HCVNeo17.wt, but was still high in cells transfected with replicons m0, m3 and m15 (Table 7). At day six, that the amount of replicon 5 RNA became undetectable in cells transfected with replicon HCVNeo17.wt, m0, and m2, but was detectable in cells transfected with replicon m3 and m15 (Table 7).

TABLE 7

Name	Hu H7	
	RNA equ.	RNA equ.
Wt	day 4	day 6
hcvneo17.m0	1 x	1 x
hcvneo17.m2	3 x	1 x
hcvneo17.m3	1 x	1 x
hcvneo17.m3	5 x	3 x
hcvneo17.m15	6 x	5 x

10

Persistence of m0, m3 and m15 replicons RNA was abolished by treatment with interferon- α or with an HCV inhibitory compound (data not shown). Moreover, RNA persistence was not observed with mutated replicons carrying the 15 NS5B GAA mutation besides adaptive mutations (data not shown). Taken together, these results demonstrated that quantitative PCR could be used to monitor replication at early times post-transfection, and can be used to evaluate the replication proficiency of replicon RNAs containing mutations.

Comparison of the results shown in Tables 6 and 7, indicated that there was a good correlation between the amount of replicon RNA detected by TaqMan and 20 the G418 cfu efficiency. Nonetheless, some mutants (m2, m3) showed a pronounced effect on G418 cfu efficiency, and little if any effect on early replication as measured by TaqMan PCR, while other mutants (m0) showed the reverse behavior.

Example 6: HCV Replicon Enhanced Cells

HCV replicon enhanced cells were produced by introducing an HCV replicon into a host, then curing the host of the replicon. Adaptive mutations (or combinations of them) by themselves increased up to 2 orders of magnitude the G418 cfu efficiency and enhanced early replication comparably. Nonetheless, even with the most effective mutants, only a small percentage of transfected cells (<5 %, data not shown) gave rise to G418 resistant clones containing functional replicons. This observation was attributed, at least in part to a low cloning efficiency of Huh-7 cells (data not shown), and only a fraction of Huh-7 cells being competent for replication.

5 Several clones were cured of endogenous replicons by treating them for about 2 weeks with IFN- α or with a HCV inhibitory compound. Analysis at the end of the treatment showed that neither viral proteins nor replicon RNA could be detected.

10 Cured cells (10IFN and Cl.60/cu) were transfected with mutated replicons and replication efficiency was determined by counting neomycin resistant clones (10IFN) or by TaqMan (10IFN and Cl.60/cu). As shown in Table 8, for all tested replicons the G418 cfu efficiency in 10IFN cells was at least 5 fold higher than in parental Huh-7 cells. This increase in G418 cfu efficiency was particularly relevant for a subset of mutants (m3, m5, m8, m9, m15).

15

20

TABLE 8

Construct	Consensus mutations			G418 cfu/10 ⁵ transfected cells
	NS3	NS5A	EMCV IRES	
pHCVNeo17.wt				12 - 56
pHCVNeo17.GAA				0
pHCVNeo17.m0		S2204R		180 - 1000
pHCVNeo17.m1		N2041T		8 - 13
pHCVNeo17.m2		S2173F		2000
pHCVNeo17.m3		S2197F		1600 - 3000
pHCVNeo17.m4		L2198S		190 - 650
pHCVNeo17.m5		K@2039		1600 - 3000
pHCVNeo17.m6	E1202G; A1347T	S2173F	extra A	600 - 2000
pHCVNeo17.m7		N2041T; S2173F		170 - 800
pHCVNeo17.m8		N2041T; S2197F		> 4000
pHCVNeo17.m9		N2041T; L2198S		1400 - 3000
pHCVNeo17.m10	E1202G	K@2039		>4000
pHCVNeo17.m11		S2197F; A2199T		>4000

TABLE 8

Construct	Consensus mutations			G418 cfu/10 ³ transfected cells
	NS3	NSSA	EMCV IRES	
pHCVNeo17.m12		N2041T; S2197F; A2199T		>4000
pHCVNeo17.m13		N2041T; S2197F	extra A	>4000
pHCVNeo17.m14		S2197F; A2199T	extra A	>4000
pHCVNeo17.m15		A2199T		> 4000

10IFN cells (2x10⁶) were transfected with 10 µg of RNA transcribed from the indicated constructs. Approximately 2x10⁵ cells were plated in a 10 cm tissue culture dish and cultured with 1 mg/ml G418 for 20 days.

5 Colonies surviving selection were stained with crystal violet and counted.

Strikingly, the best mutants yielded a number of G418 resistant clones ranging between 20 and 80% of the cell clones which grew in the absence of G418

10 (data not shown), thus indicating that the majority of 10IFN cells were competent for replication. This result was confirmed by TaqMan analysis (Table 9), in which the fold increase versus the parental Huh-7 cells was very high. The data indicates that replicons carrying adaptive mutations replicate vigorously in replicon enhanced cells such as 10IFN and Cl.60/cu.

15

TABLE 9

Name	10IFN		Cl.60/cu.	
	RNA equ.	RNA equ.	RNA equ.	RNA equ.
Wt	Day 4 1 x	day 6 1 x	day 4 1 x	Day 6 1 x
hcvneo17.m0	46 x	12 x	78 x	512 x
hcvneo17.m2	2 x	2 x	1 x	2 x
hcvneo17.m3	68 x	49 x	19 x	392 x
hcvneo17.m15	247 x	80 x	268 x	5518 x

Expression of viral proteins was determined in replicon enhanced cells

20 using an ELISA assay designed to detect the NS3 protein in transfected cells plated in 96 wells microtiter plates (Cell-ELISA). As shown in Table 10, 24 hours post-transfection cells transfected with all tested replicons expressed low but detectable levels of the NS3 protein.

TABLE 10

Name	NS3 arbitrary units			
	24 h p.t.		96 h p.t.	
	-	+ IFN	-	+IFN
Construct				
Mock	1	1	1	1
pHCVNeo17.wt	3.7	4.2	1.2	1.3
pHCVNeo17.GAA	3.1	3.2	1.1	1
pHCVNeo17.m0	3.4	3.2	9.9	0.8
pHCVNeo17.m3	5.7	4.6	4.7	1.5
pHCVNeo17.m8	6.6	5.1	15.1	1.4
pHCVNeo17.m10	8	5.6	9.2	1.8
pHCVNeo17.m11	8.4	6.2	13.6	1.8

10IFN cells (2×10^6) were transfected with 10 μ g of RNA transcribed from the indicated constructs. Cells were plated in 96 wells microtiter plates as indicated in Example 1.

5 Where indicated (+IFN), IFN- α (100 U/ml) was added to the culture medium 4 hours post-transfection. At the indicated times post-transfection, cells were fixed and analyzed by Cell-ELISA.

The early expression shown in Table 10 is likely due to translation of transfected RNA, since it was comparable in all replicons (including that carrying the GAA mutation) and was not affected by IFN- α . At 4 days post-transfection, NS3 10 expression persisted or increased in cells transfected with replicons carrying consensus mutations, but could not be detected anymore in cells transfected with wt and GAA replicons. In addition, NS3 expression was almost completely abolished when cells were cultured in the presence of IFN- α .

Taken together, these results indicated that the level of NS3 expression 15 reflected the replication rate. Indeed, NS3 expression level (Table 10) paralleled the RNA level measured by TaqMan (Table 9). The high replication proficiency of 10IFN cells was further confirmed by immunofluorescence experiments which showed that more than 50% of cells transfected with replicons m8 and m11 expressed high level of viral proteins, and that expression was almost completely abolished by 20 IFN- α .

Example 7: Replication of Full Length Constructs

This example illustrates the ability of a full length HCV genome containing adaptive mutations described herein to replicate in a replicon enhanced 25 host cell. The full length sequence of the HCV isolate Con-1 (EMBL-Genbank No. AJ238799) (plasmid pHCVRBFL.wt) and 2 derivatives containing either the N2041T

and S2173 F mutations (plasmid pHCVRBFL.m8) or the S2197F and A2199T mutations (plasmid pHCVRBFL.m11) were used as starting constructs.

5 RNAs transcribed from the starting constructs were transfected in 10IFN cells and their replication proficiency was assessed by Cell-ELISA, immunofluorescence and TaqMan. Both constructs containing consensus mutations (pHCVRBFL.m8 and pHCVRBFL.m11) replicated, while no sign of replication was observed with the wt. construct (data not shown).

Example 8: Replicons with Reporter Gene

10 This example illustrates an HCV replicon containing adaptive mutations and a reporter gene. A pHCVNeo17.wt derivative where the Neo coding region was substituted with that coding for human placental secretory alkaline phosphatase (pRBSEAP5.wt) and a derivative also containing the N2041T and S2173F mutations (plasmid pRBSEAP5.m8) were constructed. RNAs transcribed 15 from these plasmids were transfected in 10IFN cells and their replication proficiency was assessed by measuring secretion of alkaline phosphatase. Analysis of the kinetics of secretion suggested that only plasmid pRBSEAP5.m8 was competent for replication (data not shown).

20 **Example 9: SEQ. ID. Nos. 1 and 2**

SEQ. ID. NOs. 1 and 2 are provided as follows:

SEQ. ID. NO. 1

25 MSTNPKPQRKTKRNTNRRPQDVKPGGGQIVGGVYLLPRRGPRLGVRATRKT
SERSQPRGRRQPIPKARQPEGRAWAQPGYPWPLYGNEGLGWAGWLLSPRGS
RPSWGPTDPRRRSRNLGKVIDTLCGFADLMGYIPLVGAPLGAARALAHGV
RVLEDGVNYATGNLPGCSFSIFLLALLSCLTIPASAYEVNVSGVYHTVNDCS
NASIVYEAADMIMHTPGCVPCVRENNSSRCWVALPTLAARNASVPTTIRR
HVDLLVGAAALCSAMYVGDLGSVFLVAQLFTSPRRHETVQDCNCISIYPGH
30 VTGHRMAWDMMMNWSPTAALVVSQLLRIPQA VVDMVAGAHWGVLAGLA
YYSMVGNWAKVLIVMLLFAVGVDGGTYVTGGTMAKNTLGITSLFSPGSSQKIQ
LVNTNGSWHINRTALNCNDSLNTGFLAALFYVHKFNSSGCPERMASCSPIDAF
AQGWGPITYNESHSSDQRPYCWHYAPRPCGIVPAAQVCGPVYCFTPSPVVVG
TTDRFGVPTYSWGENETDVLNNNTRPPQGNWFGCTWMNSTGFTKTCGGPP

CNIGGIGNKTLTCPDCFRKHPREATYTKCGSGPWLTPLRCLVHYPYRLWHYPC
 TVNFTIFKVRMYVGGVEHRLEAACNWTRGERCNLEDRDRSESPLLLSTTEW
 QVLPCSFITLPAALSTGLIHLHQNVVDVQYLYGIGSAVVSFAIKWEYVLLFLLL
 ADARVCACLWMMMLIAQAEAALENLVLNAASVAGAHGILSFLVFFCAAWY
 5 IKGRLVPGAAAYALYGVWPLLLLLLALPPRAYAMDREMAASCAGAVFVGLILL
 TLSPHYKLFARLIWWLQYFITRAE AHLQVWIPPLNVRRGGRDAVILLTCAIHPE
 LIFTITKILLAILGPLMVLQAGITKVPYFVRAHGLIRACMLVRKVAGGHYVQM
 ALMKLAALTGTYYDHLTPLRDWAHAGLRLAVAVEPVVFSDMETKVITW
 GADTAACGDIILGLPVSARRGREIHLGPADSLEGQGWRLLAPITA YSQQTRGL
 10 LGCIITSLTGRDRNQVEGEVQVVSTATQSFLATCVNGVCWTYHGAGSKTLA
 GPKGPITQMYTNVDQDLVGWQAPPGARSLTPCTCGSSDLYLVTRHADVIPVR
 RRGDSRGSLSPRPVSYLGSSGGPLLCPSGHAVGIFRAAVCTRGVAKAVDFV
 PVESMETTMRSVPFTDNSSPPAVPQTFQVAHLHAPTGSGKSTKVPAAYAAQG
 YKVLVLPNSVAATLGFAYMSKAHGIDPNIRTGVRTITTGAPITYSTYKFLA
 15 DGGCSGGAYDIICDECHSTDSTTILGIGTVLDQAETAGARLVLVLAATPPGSV
 TVPHPNIEEVALSSTGEIPFYGKAIPETIKGGRHLIFCHSKKKCDELAAKLSGLG
 LNAVAYYRGLDVSVIPTSGDVIVVATDALMTGFTGDFDSVIDCNTCVTQTV
 FSILDPTFTIETTVPQDAVSRSQRRGRTGRGRMGIYRFVTPGERPSGMFDSSVL
 CECYDAGCAWYELTPAETSVRLLRAYLNTPLPVCQDHLEFWESVFTGLTHID
 20 AHFLSQTKQAGDNFPYLVAYQATVCARAQAPPSWDQMWKCLIRLKPTLHG
 PTPLLYRLGAVQNEVTTTHPITKYIMACMSADLEVVTSTWVLVGGVLAALAA
 YCLTTGSVVIVGRIILSGKPAIPDREVLYREFDEMEECASHLPYIEQGMQLAEQ
 FKQKAIGLLQTATKQAEAAAPVVESKWRTLEAFWAKHMWNFISGIQYLAGLS
 TLPGNPAIASLMAFTASITSPLTTQHTLLFNILGGWVAAQLAPPSAASAFVGAG
 25 IAGAAVGSIGLGKVLVDILAGYGAGVAGALVAFKVMMSGEMPSTEDLVNLLPA
 ILSPGALVVGVVCAAILRRHVGPGEGAQWMNRLIAFASRGNHVSPTHYVPE
 SDAAAARVTQILSSLTITQLKRLHQWINEDCSTPCSGSWLRDVWDWICTVLTD
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 30 RVGDFHYVTGMTTDNVKCPHQVPAPEFFTEVDGVRLHRYAPACKPLLREEV
 TFLVGLNQYLVGSQLPCEPEPDVAVLTSMLTDPSHITAETAKRRRLARGSPPSL
 ASSSASQLSAPSLKATCTTRHDSPDADLIEANLLWRQEMGGNITRVESENKVV
 ILDSFEPLQAEEDEREVSVPAEILRRSRKFPRAMPIWARPDYNPPLLESWKDPD
 YVPPVVHGCPLPPAKAPPPIPPRRKRTVVLSESTVSSALAEATKTFGSSESSA
 35 VDSGTATASPDQPSDDGDAGSDVESYSSMPPLEGEPGDPDLSDGWSVTSEE

ASEDVVCCSMSYTWTGALITPCAAEETKLPINALSNSLLRHHLVYATTSRSA
 SLRKVVTFDRLQVLDDHYRDVLKEMKAKASTVKAKLLSVEEACKLTPPHS
 ARSKFGYGAKDVRNLSSKAVNHIRSWKDLLEDTEPIDTTIMAKNEVFCVQ
 PEKGGRKPARLIVFPDLGVRVCEKMALYDVVSTLPQAVMGSSYGFQYSPGQR
 5 VEFLVNAWKAKKCPMGFYDTRCFDSTVTENDIRVEESIYQCCDLAPEARQA
 IRSLTERLYIGGPLTNSKGQNCGYRRCRASGVLTSCGNTLTCYLAAAACRA
 AKLQDCTMLVCGDDLVVVICESAGTQEDEASLRAFTEAMTRYSAPPGDPPKPE
 YDLELITSCSSNVSVAHADASGKRVYYLTRDPTTPLARAAWETARHTPVNSWL
 GNIIMYAPTLWARMILMTHFFSILLAQEQUEKALDCQIYGACYSIEPLDLPQIIQ
 10 RLHGLSAFSLHSYSPGEINRVASCLRKLGVPPLRVWRHRARSVRARLLSQGGR
 AATCGKYLFNWAVRTKLKLTPIPAASQLDLSSWFVAGYSGGDIYHSLSRARP
 RWFMWCLLLSVGVGIVLLPNR

SEQ. ID. NO. 2:

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 aatgccctatggccttcgcataatgacacccgcgttttgcactcaacggcactgagaatgacatccgtgtgaggagtcaatct
 accaatgttgacttggcccccaagccagacaggccataaggcgtcacagagcggcttacatggggccccctgat
 ctaattctaaagggcagaactgcggctatgcgggtgccgcgcagcgggtactgacgaccagctgcggtaataccctca
 catgttacttgaaggccgtcggcctgtcgagctgcgaagctccaggactgcacgatgtcgatgcggagacgaccctgt
 15 cgttatctgtgaaagcgcggggacccaagaggacgaggcggcggctacggccctacggaggctatgactagatactg
 cccccctggggacccgcccacccagaatacgcacttggagttgataacatcatgtccctccaaatgtgtcagtcgcgcacg
 atgcatactggcaaaagggtgtactatctcacccgtaccccccaccaccccttgcgcggctgcgtggagacagctagac
 acactccagtcataatccctggctaggcaacatcatgtatgcgcacccaccgtggcaaggatgtccatgtacttctt
 ctccatcccttagctcaggaacaacttggccatgttgcagatctacggggctgttactccatgtgagccacttga
 20 cctaccctcagatcatcaacgactccatggccctagcgcattttactccatgttactctccaggtaggtgagatcaatagggtggct
 tcatgcctcaggaaacttgggtaccgccttgcgagttggagacatcggccagaagtgtccgcctaggactgtcc
 cagggggggaggcgtccacttgcgtggcaagtacccttcaacttgcgtggcagaacttgcgtgggagacatata
 tgcgtccctggatgtggatgtccagctgtttgcgtttacagcgggggagacatatacagccgtctcgcc
 ccccgctggatgtggatgtccctggcctactcacttgcgttagggtaggcattactcccaaccatgt
 25 acactccaggccataggccatccctgtttttcccttt
 ttccctttttcccttgcgtggccatcttagccctaggcgtacggctagctgtgaaagggtccgtgagccgcttgc
 gctgatactggccctctgcagatcaacttgc

Other embodiments are within the following claims. While several
 30 embodiments have been shown and described, various modifications may be made
 without departing from the spirit and scope of the present invention.

WHAT IS CLAIMED IS:

1. A nucleic acid molecule comprising a region selected from the group consisting of:
 - 5 a) an altered HCV NS3 encoding region coding for one or more NS3 mutations, wherein at least one of said NS3 mutations, identified by reference to the amino acid sequence numbering of SEQ. ID. NO. 1, is selected from the group consisting of:
 - amino acid 1095 being Ala,
 - 10 amino acid 1202 being Gly, and
 - amino acid 1347 being Thr;
 - b) an altered HCV NS5A encoding region coding for one or more NS5A mutations, wherein at least one of said NS5A mutations, identified by reference to the amino acid sequence numbering of SEQ. ID. NO. 1, is selected from the group consisting of:
 - 15 amino acid 2041 being Thr,
 - a Lys insertion between residue 2039 and 2040.
 - amino acid 2173 being Phe,
 - amino acid 2197 being Phe,
 - 20 amino acid 2198 being Ser,
 - amino acid 2199 being Thr, and
 - amino acid 2204 being Arg; and
 - c) an altered encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) region containing one or more EMCV IRES mutations,
 - 25 wherein at least one of said EMCV IRES mutations, identified by reference to the nucleotide number of SEQ. ID. NO. 3, is an insertion at nucleotide 1736 of adenine.
2. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule comprises said NS5A encoding region.
- 30 3. The nucleic acid molecule of claim 2, wherein at least two of said NS5A adaptive mutations are present.

4. The nucleic acid molecule of claim 2, further comprising a region encoding for a HCV NS3 region, wherein said NS3 region may be the same or different than said altered NS3 region.

5 5. The nucleic acid molecule of claim 4, wherein said nucleic acid molecule is an HCV replicon comprising a HCV 5' UTR-PC region, said NS3 encoding region, an HCV NS4A encoding region, an HCV NS4B encoding region, said NS5A encoding region, an HCV NS5B encoding region, and a HCV 3' UTR.

10 6. The nucleic acid molecule of claim 5, wherein said HCV replicon further comprises a sequence encoding for a reporter protein.

7. The nucleic acid molecule of claim 5, wherein said HCV replicon further comprises a sequence encoding for a selection protein.

15 8. The nucleic acid molecule of claim 5, wherein said HCV replicon further comprises a HCV core encoding region, a HCV E1 encoding region, a HCV E2 encoding region, a HCV p7 encoding region, and a HCV NS2 encoding region.

20 9. A nucleic acid molecule comprising a region selected from the group consisting of:

25 a) an altered HCV NS3 encoding region containing one or more NS3 mutations, wherein at least one of said NS3 mutations, identified by reference to the nucleotide numbering of SEQ. ID. NO. 2, is selected from the group consisting of:

nucleotide 3625 being cytosine,
nucleotide 3946 being guanine,
nucleotide 4380 being adenine,

30 b) an altered HCV NS5A encoding region containing one or more NS5A mutations, wherein at least one of said NS5A mutations, identified by reference to the nucleotide numbering of SEQ. ID. NO. 2, is selected from the group consisting of:

an insertion of 3 adenine residues between nucleotide 6458 and 6459,
nucleotide 6463 being cytosine,
35 nucleotide 6859 being thymine or uracil,

nucleotide 6931 being thymine or uracil,
nucleotide 6934 being cytosine,
nucleotide 6936 being adenine, and
nucleotide 6953 being adenine or guanine; and

5 c) an altered encephalomyocarditis virus (EMCV) internal
ribosome entry site (IRES) region containing one or more EMCV IRES mutations,
wherein at least one of said EMCV IRES mutations, identified by reference to the
nucleotide number of SEQ. ID. NO. 3, is an insertion at nucleotide 1736 of adenine.

10 10. The nucleic acid molecule of claim 9, wherein said molecule
comprises said altered NS5A encoding region, and the nucleotide sequence of said
altered NS5A region is provided for by bases 6258-7598 of SEQ. ID. NO. 2, or the
RNA version thereof, modified with one or more of said NS5A modifications selected
from the group consisting of:
15 an insertion of 3 adenine residues between nucleotide 6458 and 6459,
nucleotide 6463 being cytosine,
nucleotide 6859 being thymine or uracil,
nucleotide 6931 being thymine or uracil,
nucleotide 6934 being cytosine,
20 nucleotide 6936 being adenine, and
nucleotide 6953 being adenine or guanine.

11. The nucleic acid molecule of claim 10, wherein said molecule
is an HCV replicon comprising a HCV 5' UTR-PC region, a modified HCV NS3-
25 NS5B region, and a HCV 3' UTR, wherein said modified NS3-NS5B region
comprises said altered NS5A region.

12. The nucleic acid molecule of claim 11, wherein said 5' UTR-
PC region is the RNA version of bases 1-377 of SEQ. ID. NO. 2 and said 3' UTR is
30 the RNA version of bases 9374-9605 of SEQ. ID. NO. 2.

13. The nucleic acid molecule of claim 10, wherein said molecule
is an HCV replicon comprising a HCV 5' UTR-PC region, a modified HCV NS3-
NS5B region, and a HCV 3' UTR, wherein
35 said 5' UTR-PC region is the RNA version of bases 1-377 of SEQ. ID. NO. 2;

said 3' UTR is the RNA version of bases 9374-9605 of SEQ. ID. NO. 2; and said modified NS3-NS5B region consists of the RNA version of bases 3420-9371 of SEQ. ID. NO. 2 modified with one or more modifications selected from the group consisting of:

- 5 nucleotide 4380 being adenine,
 nucleotide 3625 being cytosine,
 nucleotide 3946 being guanine,
 an insertion of 3 adenine residues between nucleotide 6458 and nucleotide 6459,
 nucleotide 6463 being cytosine,
- 10 nucleotide 6859 being uracil,
 nucleotide 6931 being uracil,
 nucleotide 6934 being cytosine,
 nucleotide 6936 being adenine, and
 nucleotide 6953 being adenine or guanine.

15

14. The nucleic acid molecule of claim 13, wherein said replicon is a genomic replicon that further comprises the RNA version of nucleotides 378-3419 of SEQ. ID. NO. 2.

- 20 15. A nucleic acid molecule comprising the nucleic acid base sequence of bases 1-7989 of SEQ. ID. NO. 3, or the RNA version thereof, consisting of one or more different modifications selected from the group consisting of:
 - a) nucleotides 5335-5337 modified to code for arginine;
 - b) nucleotides 5242-5244 modified to code for phenylalanine;
 - c) nucleotides 5314-5316 modified to code for phenylalanine;
 - d) nucleotides 5317-5319 modified to code for serine;
 - e) nucleotides coding for lysine inserted after nucleotide 4843;
 - f) nucleotides 2329-2331 modified to code for glycine, nucleotides 2764-2766 modified to code for threonine, nucleotides 5242-5244 modified to code for phenylalanine, and an extra adenosine inserted after nucleotide 1736;
 - g) nucleotides 4846-4848 modified to code for threonine, and nucleotides 5242-5244 modified to code for phenylalanine;
 - h) nucleotides 4846-4848 modified to code for threonine, and nucleotides 5314-5316 modified to code for phenylalanine;

- i) nucleotides 4846-4848 modified to code for threonine, and nucleotides 5317-5319 modified to code for serine;
- j) nucleotides 2329-2331 modified to code for glycine, and nucleotides coding for lysine inserted after nucleotides 4843;
- 5 k) nucleotides 5314-5316 modified to code for phenylalanine and nucleotides 5320-5322 modified to code for threonine;
- l) nucleotides 4846-4848 modified to code for threonine, nucleotides 5314-5316 modified to code for phenylalanine, and nucleotides 5320-5322 modified to code for threonine;
- 10 m) nucleotides 4846-4848 modified to code for threonine, nucleotides 5314-5316 modified to code for phenylalanine, and an extra adenosine inserted after nucleotide 1736; and
- n) nucleotides 5314-5316 modified to code for phenylalanine, nucleotides 5320-5322 modified to code for threonine, and an extra adenosine inserted after nucleotide 1736;
- 15 and
- o) nucleotides 5320-5322 modified to code for threonine.

16. The nucleic acid of claim 15, wherein said one or more different modifications is selected from the group consisting of:

- 20 a) C5337A;
- b) C5243T or U;
- c) C5315T or U;
- d) T or U5318C;
- e) AAA inserted after 4843;
- 25 f) A2330G, G2764A, C5243T or U, and adenosine inserted 1736;
- g) A4847C and C5243T or U;
- h) A4847C and C5315T or U;
- i) A4847C and T or U5318C;
- j) A2330G and AAA inserted after 4843;
- 30 k) C5315T or U and G5320A;
- l) A4847C, C5315T or U, and G5320A;
- m) A4847C, C5315T or U, and adenosine inserted 1736;
- n) C5315T or U, G5320A and adenosine inserted 1736; and
- o) G5320A.

17. The nucleic acid of claim 16, wherein said nucleic acid is RNA and comprises said nucleic acid base sequence.

5 18. The nucleic acid of claim 17, wherein said nucleic acid is RNA and consists of said nucleic acid base sequence.

10 19. An expression vector comprising a nucleotide sequence coding for the nucleic acid molecule of any one of claims 1-18, wherein said nucleotide sequence is transcriptionally coupled to an exogenous promoter.

20. A recombinant cell human hepatoma cell, wherein said cell comprises the nucleic acid of any one of claims 5-8 and 11-18.

15 21. The recombinant cell of claim 20, wherein said hepatoma cell is an Huh-7 cell.

20 22. The recombinant cell of claim 20, wherein said cell is derived from a Huh-7 cell.

23. A recombinant cell made by a process comprising the step of introducing into a human hepatoma cell the nucleic acid of any one of claims 5-8 and 11-18.

25 24. A method of making an HCV replicon enhanced cell comprising the steps of:

a) introducing and maintaining a HCV replicon in a cell; and
b) curing said cell of said HCV replicon to produce said replicon enhanced cell.

30 25. The method of claim 24, wherein said cell is a human hepatoma cell.

35 26. The method of claim 24, wherein said cell is a Huh-7 cell or is derived from a Huh-7 cell.

27. The method of claim 26, further comprising the step of confirming the ability of said replicon enhanced cell to maintain an HCV replicon.

5 28. A method of making an HCV replicon enhanced cell containing a functional HCV replicon comprising the steps of:

- a) introducing and maintaining a first HCV replicon in a cell;
- b) curing said cell of said first replicon to produce a cured cell;

and

10 c) introducing and maintaining a second HCV replicon into said cured cell, wherein said second HCV replicon may be the same or different than said first HCV replicon.

15 29. The method of claim 28, wherein said cell is a human hepatoma cell.

30. The method of claim 29, wherein said human hepatoma cell is a Huh-7 cell.

20 31. The method of claim 30, wherein said human hepatoma cell is derived from a Huh-7 cell.

32. An HCV replicon enhanced cell made by the method of any one of claims 24-27.

25 33. An HCV replicon enhanced cell containing a HCV replicon made by the method of any one of claims 28-31.

30 34. A method of measuring the ability of a compound to affect HCV activity comprising the steps of:

- a) providing said compound to the HCV replicon enhanced cell of claim 33; and
- b) measuring the ability of said compound to effect one or more replicon activities as a measure of the effect on HCV activity.

35

35. The method of claim 34, wherein said compound is a ribozyme.
36. The method of claim 34, wherein said compound is an antisense nucleic acid.
5
37. The method of claim 34, wherein compound is an organic compound.
38. The method of claim 34, wherein said step (b) measures HCV
10 protein production.
39. The method of claim 33, wherein said step (b) measures production of RNA transcripts.

1 GCCAGCCCCC GATTGGGGC GACACTCCAC CATAGATCAC TCCCCGTGA
51 GGAACTACTG TCTTCACGCA GAAAGCGTCT AGCCATGGCG TTAGTATGAG
101 TGTCTGCAG CCTCCAGGAC CCCCCCTCCC GGGAGAGCCA TAGTGGTCTG
151 CGGAACCGGT GAGTACACCG GAATTGCCAG GACGACCGGG TCCTTTCTTG
201 GATCAACCCG CTCAATGCCT GGAGATTGG GCGTGCCCCC GCGAGACTGC
251 TAGCCGAGTA GTGTTGGTC GCGAAAGGCC TTGTGGTACT GCCTGATAGG
301 GTGCTTGCGA GTGCCCGGG AGGTCTCGTA GACCGTGCAC CATGAGCAGC
351 AATCCTAAAC CTCAAAGAAA AACCAAAGGG CGCGCCATGA TTGAACAAGA
401 TGGATTGCAC GCAGGTTCTC CGGCCGCTTG GGTGGAGAGG CTATTCGGCT
451 ATGACTGGGC ACAACAGACA ATCGGCTGCT CTGATGCCGC CGTGTCCGG
501 CTGTCAGCGC AGGGGCGCCC GGTTCTTTT GTCAAGACCG ACCTGTCCGG
551 TGCCCTGAAT GAACTGCAGG ACGAGGCAGC GCGGCTATCG TGGCTGGCCA
601 CGACGGCGT TCCTTGCGCA GCTGTGCTCG ACGTTGTCAC TGAAGCGGGA
651 AGGGACTGGC TGCTATTGGG CGAAGTGCCG GGGCAGGATC TCCTGTCATC
701 TCACCTTGCT CCTGCCGAGA AAGTATCCAT CATGGCTGAT GCAATGCGGC
751 GGCTGCATAC GCTTGATCCG GCTACCTGCC CATTGACCA CCAAGCGAAA
801 CATCGCATCG AGCGAGCACG TACTCGGATG GAAGCCGGTC TTGTCGATCA
851 GGATGATCTG GACGAAGAGC ATCAGGGCT CGCGCCAGCC GAACTGTTCG
901 CCAGGCTCAA GGCGCGCATG CCCGACGGCG AGGATCTCGT CGTGACCCAT
951 GGCGATGCCT GCTTGCCGAA TATCATGGTG GAAAATGGCC GCTTTCTGG
1001 ATTCAATCGAC TGTGGCCGGC TGGGTGTGGC GGACCGCTAT CAGGACATAG
1051 CGTTGGCTAC CCGTGATATT GCTGAAGAGC TTGGCGGCAGA ATGGGCTGAC
1101 CGCTTCTCG TGCTTTACGG TATCGCCGCT CCCGATTGCG AGCGCATCGC
1151 CTTCTATCGC CTTCTTGACG AGTTCTCTG AGTTAAACA GACCACAAAG
1201 GTTTCCCTCT AGCGGGATCA ATTCCGGCCC TCTCCCTCCC CCCCCCTAA
1251 CGTTACTGGC CGAAGCCGCT TGGAAATAAGG CCGGTGTGCG TTTGTCTATA
1301 TGTTATTTTC CACCATATTG CCGTCTTTG GCAATGTGAG GGCCCGGAAA
1351 CCTGGCCCTG TCTTCTTGAC GAGCATTCT AGGGGTCTTT CCCCTCTCGC
1401 CAAAGGAATG CAAGGTCTGT TGAATGTGCG GAAGGAAGCA GTTCCTCTGG
1451 AAGCTTCTTG AAGACAAACA ACGTCTGTAG CGACCCCTTG CAGGCAGCGG
1501 AACCCCCCAC CTGGCGACAG GTGCCTCTGC GGCCAAAAGC CACGTGTATA

FIG. 1A

1551 AGATACACCT GCAAAGGCGG CACAACCCA GTGCCACGTT GTGAGTTGGA
 1601 TAGTTGTGGA AAGAGTCAAA TGGCTCTCCT CAAGCGTATT CAACAAGGGG
 1651 CTGAAGGATG CCCAGAAGGT ACCCCATTGT ATGGGATCTG ATCTGGGCC
 1701 TCGGTGCACA TGCTTTACAT GTGTTAGTC GAGGTTAAAA AACGTCTAGG
 1751 CCCCCCGAAC CACGGGGACG TGGTTTCCT TTGAAAACA CGATAATACC
 1801 ATGGCGCCTA TTACGGCCTA CTCCCAACAG ACGCGAGGCC TACTTGGCTG
 1851 CATCATCACT AGCCTCACAG GCCGGGACAG GAACCAGGTC GAGGGGGAGG
 1901 TCCAAGTGGT CTCCACCGCA ACACAATCTT TCCTGGCGAC CTGCGTCAAT
 1951 GGCGTGTGTT GGACTGTCTA TCATGGTGCC GGCTCAAAGA CCCTTGCCGG
 2001 CCCAAAGGGC CCAATCACCC AAATGTACAC CAATGTGGAC CAGGACCTCG
 2051 TCGGCTGGCA AGCGCCCCC GGGGCGCGTT CCTTGACACC ATGCACCTGC
 2101 GGCAGCTCGG ACCTTTACTT GGTCACGAGG CATGCCGATG TCATTCCGGT
 2151 GCGCCGGCGG GGCGACAGCA GGGGGAGCCT ACTCTCCCCC AGGCCCGTCT
 2201 CCTACTTGAA GGGCTCTTCG GGCGGTCCAC TGCTCTGCC CTCGGGCAC
 2251 GCTGTGGCA TCTTCGGGC TGCCGTGTGC ACCCGAGGGG TTGCGAAGGC
 2301 GGTGGACTTT GTACCCGTG AGTCTATGGA AACCACTATG CGGTCCCCGG
 2351 TCTTCACGGA CAACTCGTCC CCTCCGGCCG TACCGCAGAC ATTCCAGGTG
 2401 GCCCATCTAC ACGCCCCTAC TGGTAGCGGC AAGAGCACTA AGGTGCCGGC
 2451 TGCCTATGCA GCCCAAGGGT ATAAGGTGCT TGTCCTGAAC CCGTCCGTG
 2501 CCGCCACCCCT AGGTTTCGGG GCGTATATGT CTAAGGCACA TGGTATCGAC
 2551 CCTAACATCA GAACCGGGGT AAGGACCATC ACCACGGGTG CCCCCATCAC
 2601 GTACTCCACC TATGGCAAGT TTCTTGCCGA CGGTGGTTGC TCTGGGGCG
 2651 CCTATGACAT CATAATATGT GATGAGTGCC ACTCAACTGA CTCGACCACT
 2701 ATCCTGGGCA TCGGCACAGT CCTGGACCAA GCGGAGACGG CTGGAGCGCG
 2751 ACTCGTCGTG CTCGCCACCG CTACGCCCTCC GGGATCGGTC ACCGTGCCAC
 2801 ATCCAAACAT CGAGGAGGTG GCTCTGTCCA GCACTGGAGA AATCCCCTTT
 2851 TATGGCAAAG CCATCCCCAT CGAGACCATC AAGGGGGGGGA GGCACCTCAT
 2901 TTTCTGCCAT TCCAAGAAGA AATGTGATGA GCTCGCCGCG AAGCTGTCCG
 2951 GCCTCGGACT CAATGCTGTA GCATATTACC GGGGCCTTGA TGTATCCGTC
 3001 ATACCAAACCA GCGGAGACGT CATTGTCGTA GCAACGGACG CTCTAATGAC
 3051 GGGCTTTACC GGCGATTTCG ACTCAGTGAT CGACTGCAAT ACATGTGTCA

FIG. 1B

3101 CCCAGACAGT CGACTTCAGC CTGGACCCGA CCTTCACCAT TGAGACGACG
3151 ACCGTGCCAC AAGACGCGGT GTCACGCTCG CAGCGCGAG GCAGGACTGG
3201 TAGGGGCAGG ATGGGCATTT ACAGGTTGT GACTCCAGGA GAACGGCCCT
3251 CGGGCATGTT CGATTCTCG GTTCTGTGCG AGTGCTATGA CGCGGGCTGT
3301 GCTTGGTACG AGCTCACGCC CGCCGAGACC TCAGTTAGGT TGCGGGCTTA
3351 CCTAAACACA CCAGGGTTGC CCGTCTGCCA GGACCATCTG GAGTTCTGGG
3401 AGAGCGTCTT TACAGGCCTC ACCCACATAG ACGCCCATT TTTGTCCCAG
3451 ACTAACAGG CAGGAGACAA CTTCCCTAC CTGGTAGCAT ACCAGGCTAC
3501 GGTGTGCGCC AGGGCTCAGG CTCCACCTCC ATCGTGGGAC CAAATGTGGA
3551 AGTGTCTCAT ACGGCTAAAG CCTACGCTGC ACGGGCAAC GCCCCTGCTG
3601 TATAGGCTGG GAGCCGTTCA AAACGAGGTT ACTACCACAC ACCCCATAAC
3651 CAAATACATC ATGGCATGCA TGTCGGCTGA CCTGGAGGTC GTCACGAGCA
3701 CCTGGGTGCT GGTAGGCGGA GTCCTAGCAG CTCTGGCCGC GTATTGCCTG
3751 ACAACAGGCA GCGTGGTCAT TGTGGCAGG ATCATCTTGT CCGGAAAGCC
3801 GGCCATCATT CCCGACAGGG AAGTCCTTA CCGGGAGTTC GATGAGATGG
3851 AAGAGTGCAG CTCACACCTC CCTTACATCG AACAGGGAAT GCAGCTGCC
3901 GAACAATTCA AACAGAAGGC AATCGGGTTG CTGCAAACAG CCACCAAGCA
3951 AGCGGAGGCT GCTGCTCCCG TGGTGGAAATC CAAGTGGCGG ACCCTCGAAG
4001 CCTTCTGGGC GAAGCATATG TGGATTTCAT TCAGCGGGAT ACAATATTTA
4051 GCAGGCTTGT CCACTCTGCC TGGCAACCCC GCGATAGCAT CACTGATGGC
4101 ATTACACAGCC TCTATCACCA GCCCGCTCAC CACCCAACAT ACCCTCTGT
4151 TTAACATCCT GGGGGGATGG GTGGCCGCC AACTTGCTCC TCCCAGCGCT
4201 GCTTCTGCTT TCGTAGGCGC CGGCATCGCT GGAGCGGCTG TTGGCAGCAT
4251 AGGCCTTGGG AAGGTGCTTG TGGATATTTT GGCAGGTTAT GGAGCAGGGG
4301 TGGCAGGCGC GCTCGTGGCC TTTAAGGTCA TGAGCGCGA GATGCCCTCC
4351 ACCGAGGACC TGGTTAACCT ACTCCCTGCT ATCCTCTCCC CTGGCGCCCT
4401 AGTCGTGGG GTCGTGTGCG CAGCGATACT GCGTCGGCAC GTGGGCCCAG
4451 GGGAGGGGGC TGTGCAGTGG ATGAACCGGC TGATAGCGTT CGCTTGGCGG
4501 GGTAACCACG TCTCCCCAC GCACTATGTG CCTGAGAGCG ACGCTGCAGC
4551 ACGTGTCACT CAGATCCTCT CTAGTCTTAC CATCACTCAG CTGCTGAAGA
4601 GGCTTCACCA GTGGATCAAC GAGGACTGCT CCACGCCATG CTCCGGCTCG

FIG. 1C

4651 TGGCTAAGAG ATGTTGGGA TTGGATATGC ACGGTGTTGA CTGATTCAA
4701 GACCTGGCTC CAGTCCAAGC TCCTGCCGCG ATTGCCGGGA GTCCCCCTCT
4751 TCTCATGTCA ACGTGGGTAC AAGGGAGTCT GGCGGGGCGA CGGCATCATG
4801 CAAACCACCT GCCCATGTGG AGCACAGATC ACCGGACATG TGAAAAACGG
4851 TTCCATGAGG ATCGTGGGGC CTAGGACCTG TAGTAACACG TGGCATGGAA
4901 CATTCCCCAT TAACGCGTAC ACCACGGGC CCTGCACGCC CTCCCCGGCG
4951 CCAAATTATT CTAGGGCGCT GTGGCGGGTG GCTGCTGAGG AGTACGTGGA
5001 GGTTACGCCG GTGGGGGATT TCCACTACGT GACGGGCATG ACCACTGACA
5051 ACGTAAAGTG CCCGTGTCAG GTTCCGGCCC CCGAATTCTT CACAGAAAGTG
5101 GATGGGGTGCG GGTTGCACAG GTACCGCTCCA GCGTGCAAAC CCCTCCTACG
5151 GGAGGAGGTC ACATTCTGG TCGGGCTCAA TCAATACCTG GTTGGGTAC
5201 AGCTCCCAG CGAGCCCGAA CCGGACGTAG CAGTGCTCAC TTCCATGCTC
5251 ACCGACCCCT CCCACATTAC GGCAGAGACG GCTAACCGTA GGCTGCCAG
5301 GGGATCTCCC CCCTCCTTGG CCAGCTCATC AGCTAGCCAG CTGTCTGCGC
5351 CTTCCTTGAA GGCAACATGC ACTACCCGTC ATGACTCCCC GGACGCTGAC
5401 CTCATCGAGG CCAACCTCCT GTGGCGGCAG GAGATGGCG GGAACATCAC
5451 CCGCGTGGAG TCAGAAAATA AGGTAGTAAT TTTGGACTCT TTGAGGCCGC
5501 TCCAAGCGGA GGAGGATGAG AGGGAAGTAT CCGTTCCGGC GGAGATCCTG
5551 CGGAGGTCCA GGAAATTCCC TCGAGCGATG CCCATATGGG CACGCCCGA
5601 TTACAACCCCT CCACTGTTAG AGTCCTGGAA GGACCCGGAC TACGTCCCTC
5651 CAGTGGTACA CGGGTGTCCA TTGCCGCCTG CCAAGGCCCC TCCGATAACCA
5701 CCTCCACCGA GGAAGAGGAC GGTTGTCCTG TCAGAATCTA CCGTGTCTTC
5751 TGCCCTGGCG GAGCTCGCCA CAAAGACCTT CCGCAGCTCC GAATCGTCGG
5801 CCGTCGACAG CGGCACGGCA ACGGCCTCTC CTGACCAGCC CTCCGACGAC
5851 GGCGACGCCG GATCCGACGT TGAGTCGTAC TCCTCCATGC CCCCCCTTGA
5901 GGGGGAGCCG GGGGATCCCG ATCTCAGCGA CGGGTCTTGG TCTACCGTAA
5951 GCGAGGAGGC TAGTGAGGAC GTCGTCTGCT GCTCGATGTC CTACACATGG
6001 ACAGGCGCCC TGATCACGCC ATGCGCTGCG GAGGAAACCA AGCTGCCCAT
6051 CAATGCACTG AGCAACTCTT TGCTCCGTCA CCACAACTTG GTCTATGCTA
6101 CAACATCTCG CAGCGCAAGC CTGCGGCAGA AGAAGGTCAC CTTTGACAGA
6151 CTGCAGGTCC TGGACGACCA CTACCGGGAC GTGCTCAAGG AGATGAAGGC

FIG. 1D

6201 GAAGGCGTCC ACAGTTAAGG CTAAACTTCT ATCCGTGGAG GAAGCCTGTA
6251 AGCTGACGCC CCCACATTG GCCAGATCTA AATTGGCTA TGGGGCAAAG
6301 GACGTCCCGA ACCTATCCAG CAAGGCCGTT AACCACATCC GCTCCGTGTG
6351 GAAGGACTTG CTGGAAGACA CTGAGACACC AATTGACACC ACCATCATGG
6401 CAAAAAAATGA GGTTTCTGC GTCCAACCAG AGAAGGGGGG CCGCAAGCCA
6451 GCTCGCCTTA TCGTATTCCC AGATTGGGG GTTCGTGTGT GCGAGAAAAT
6501 GGCCCTTTAC GATGTGGTCT CCACCCCTCCC TCAGGCCGTG ATGGGCTCTT
6551 CATA CGGATT CCAATACTCT CCTGGACAGC GGGTCGAGTT CCTGGTGAAT
6601 GCCTGGAAAG CGAAGAAATG CCCTATGGGC TTCGCATATG ACACCCGCTG
6651 TTTTGACTCA ACGGTCACTG AGAATGACAT CCGTGTGAG GAGTCAATCT
6701 ACCAATGTTG TGACTTGGCC CCCGAAGCCA GACAGGCCAT AAGGTCGCTC
6751 ACAGAGCGGC TTTACATCGG GGGCCCCCTG ACTAATTCTA AAGGGCAGAA
6801 CTGCGGCTAT CGCCGGTGCC GCGCGAGCGG TGTACTGACG ACCAGCTGCG
6851 GTAATACCCCT CACATGTTAC TTGAAGGCCG CTGCGGCCCTG TCGAGCTGCG
6901 AAGCTCCAGG ACTGCACGAT GCTCGTATGC GGAGACGACC TTGTCGTTAT
6951 CTGTGAAAGC GCGGGGACCC AAGAGGACGA GCGGAGCCTA CGGGCCTTCA
7001 CGGAGGCTAT GACTAGATAC TCTGCCCCC CTGGGGACCC GCCCAAACCA
7051 GAATACGACT TGGAGTTGAT AACATCATGC TCCTCCAATG TGTCAGTCGC
7101 GCACGATGCA TCTGGAAAAA GGGTGTACTA TCTCACCCGT GACCCACCA
7151 CCCCCCTTGC GCGGGCTGCG TGGGAGACAG CTAGACACAC TCCAGTCAAT
7201 TCCTGGCTAG GCAACATCAT CATGTATGCG CCCACCTTGT GGGCAAGGAT
7251 GATCCTGATG ACTCATTCT TCTCCATCCT TCTAGCTCAG GAACAATTG
7301 AAAAGCCCT AGATTGTCAG ATCTACGGGG CCTGTTACTC CATTGAGCCA
7351 CTTGACCTAC CTCAGATCAT TCAACGACTC CATGGCCTTA GCGCATTTTC
7401 ACTCCATAGT TACTCTCCAG GTGAGATCAA TAGGGTGGCT TCATGCCTCA
7451 GGAAACTTGG GGTACCGCCC TTGCGAGTCT GGAGACATCG GGCCAGAAGT
7501 GTCCCGCGCTA GGCTACTGTC CCAGGGGGGG AGGGCTGCCA CTTGTGGCAA
7551 GTACCTCTTC AACTGGGCAG TAAGGACCAA GCTCAAACTC ACTCCAATCC
7601 CGGCTGCGTC CCAGTTGGAT TTATCCAGCT GGTTCGTTGC TGGTTACAGC
7651 GGGGGAGACA TATATCACAG CCTGTCTCGT GCCCGACCCC GCTGGTTCAT
7701 GTGGTGCCTA CTCCCTACTTT CTGTAGGGGT AGGCATCTAT CTACTCCCCA

FIG. 1E

7751 ACCGATGAAC GGGGAGCTAA ACACTCCAGG CCAATAGGCC ATCCTGTTT
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 7851 TTCTCCTTTT TTTTCCTCT TTTTTTCCTT TTCTTCCTT TGGTGGCTCC
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 8201 ATCGTCCAAC GCACGGGGAG AGGGGGTTG CGTATTGGC GCACTTCCGC
 8251 TTCCCTCGCTC ACTGACTCGC TGCGCTCGTT CGTTGGCCTG CGGCGAGCCG
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 8551 TGTTCCGACC CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG
 8601 GAAGCGTGGC GCTTTCTCAT AGCTCACGCT GTAGGTATCT CAGTCGGTG
 8651 TAGTCGTTG CTCAGGAGCT GGGCTGTGTG CACGAACCCC CCGTCAGCC
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 9151 CTAAAGTATA TATGAGTAAA CTTGGTCTGA CAGTTACCAA TGCTTAATCA
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 9251 TGACTCCCCG TCGTGTAGAT AACTACGATA CGGGAGGGCT TACCATCTGG

FIG. 1F

9301 CCCCAGTGCT GCAATGATA CCGGAGAAC ACGTCACCC GCACCAGATT
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 10501 CCGCAACTGT TGGGAAGGGC GGTCAGTACG CGCTTCTTCG CTATTACGCC
 10551 AACTGGCGAA AGGGGGATGT GCTGCAAGGC GATTAAGTTG GGTAACGCCA
 10601 GGGTTTCCC AATCACGACG TTGTAAAACG ACAGCCAATG AATTGAAGCT
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Fig. 1G

IAP12 Rec'd PCT/PTO 01 MAY 2006

SEQUENCE LISTING

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WO 2002/059321 A3

(54) Title: HEPATITIS C VIRUS REPLICONS AND REPLICON ENHANCED CELLS

(57) Abstract: The present invention features nucleic acid containing one or more adaptive mutations, and HCV replicon enhanced cells. Adaptive mutations are mutations that enhance HCV replicon activity. HCV replicon enhanced cells are cells having an increased ability to maintain an HCV replicon.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/00526

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7	C12N15/40	C12N15/51	C12N5/10	C07K14/18	C12N15/85
	C12N7/00	G01N33/50			

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, Sequence Search, CHEM ABS Data, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 1995, no. 02, 31 March 1995 (1995-03-31) & JP 06 319583 A (SOUYAKU GIJUTSU KENKYUSHO:KK), 22 November 1994 (1994-11-22) abstract -& DATABASE GENESEQ [Online] E.B.I. Hinxton U.K.; 6 December 1995 (1995-12-06), SOYAKU GIJUTSU KENKYUSHO KK.: "Hepatitis C virus RNA helicase" XP002245688 Database accession no. AAR68864 Ala at amino acid 1095 abstract ----- -/-	1,9,19
X		1,9,19

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the International filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
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"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the International search

2 October 2003

Date of mailing of the International search report

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/00526

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE EM VI [Online] E.B.I. HINXTON U.K.; 27 April 2000 (2000-04-27), NAGAYAMA K ET AL: "Hepatitis C virus strain MD27 complete genome" XP002245689 Database accession no. AF207768 abstract</p> <p>-----</p>	1, 9, 19
A	<p>EP 1 043 399 A (BARTENSCHLAGER RALF DR) 11 October 2000 (2000-10-11) cited in the application claims 22-30</p> <p>-----</p>	
A	<p>BLIGHT K J ET AL: "EFFICIENT INITIATION OF HCV RNA REPLICATION IN CELL CULTURE" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE., US, vol. 290, 8 December 2000 (2000-12-08), pages 1972-1974, XP002951271 ISSN: 0036-8075</p> <p>-----</p>	
A	<p>LOHMANN V ET AL: "REPLICATION OF SUBGENOMIC HEPATITIS C VIRUS RNAs IN A HEPATOMA CELLLINE" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE., US, vol. 285, 2 July 1999 (1999-07-02), pages 110-113, XP000960693 ISSN: 0036-8075 cited in the application</p> <p>-----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/00526

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
JP 06319583	A 22-11-1994	NONE		
EP 1043399	A 11-10-2000	DE 19915178 A1 AT 236988 T AU 2518000 A CA 2303526 A1 DE 50001673 D1 DK 1043399 T3 EP 1043399 A2 JP 2001017187 A US 6630343 B1		05-10-2000 15-04-2003 19-10-2000 03-10-2000 15-05-2003 28-07-2003 11-10-2000 23-01-2001 07-10-2003

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/00526

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1, 9, 19 all partially

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 9, 19 (all partially)

A nucleic acid molecule comprising an altered HCV NS3 encoding region coding for one or more NS3 mutations, wherein at least one of said NS3 mutations, identified by reference to the amino acid sequence numbering of SeqIdNo.1, is amino acid 1095 being Ala; A nucleic acid molecule comprising an altered HCV NS3 encoding region coding for one or more NS3 mutations, wherein at least one of said NS3 mutations, identified by reference to the nucleotide sequence numbering of SeqIdNo.2, is nucleotide 3625 being cytosine; an expression vector comprising said nucleic acid molecule.

2. claims: 1, 9, 15-19 (all partially)

As for invention 1, wherein said at least one of said NS3 mutations is amino acid 1202 being Gly / nucleotide 3946 of SeqIdNo.2 being guanine / nucleotide 2330 of SeqIdNo.3 being guanine.

3. claims: 1, 9, 15-19 (all partially)

As for invention 1, wherein said at least one of said NS3 mutations is amino acid 1347 being Thr / nucleotide 4380 of SeqIdNo.2 being alanine / nucleotide 2764 of SeqIdNo.3 being alanine.

4. claims: 1-23 (all partially)

A nucleic acid molecule comprising an altered HCV NS5 encoding region coding for one or more NS5 mutations, wherein at least one of said NS5 mutations, identified by reference to the amino acid sequence numbering of SeqIdNo.1, is amino acid 2041 being Thr; A nucleic acid molecule comprising an altered HCV NS5 encoding region coding for one or more NS5 mutations, wherein at least one of said NS5 mutations, identified by reference to the nucleotide sequence numbering of SeqIdNo.2, is nucleotide 6463 being cytosine / nucleotide 4847 of SeqIdNo.3 being cytosine; said nucleic acid being an HCV replicon; expression vector and recombinant cell comprising said nucleic acid molecule.

5. claims: 1-23 (all partially)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

As for invention 4, wherein said at least one of said NS5 mutations is Lys insertion between residue 2039 and 2040 of SeqIdNo.1 / insertion of 3 adenine residues between nucleotide 6458 and 6459 of SeqIdNo.2 / insertion of 3 adenine residues after nucleotide 4843 of SeqIdNo.3

6. claims: 1-23 (all partially)

As for invention 4, wherein said at least one of said NS5 mutations is amino acid 2173 being Phe / nucleotide 6859 of SeqIdNo.2 being thymine or uracil / nucleotide 5243 of SeqIdNo.3 being thymine or uracil.

7. claims: 1-23 (all partially)

As for invention 4, wherein said at least one of said NS5 mutations is amino acid 2197 being Phe / nucleotide 6931 of SeqIdNo.2 being thymine or uracil / nucleotide 5315 of SeqIdNo.3 being thymine or uracil.

8. claims: 1-23 (all partially)

As for invention 4, wherein said at least one of said NS5 mutations is amino acid 2198 being Ser / nucleotide 6934 of SeqIdNo.2 being cytosine / nucleotide 5318 of SeqIdNo.3 being cytosine.

9. claims: 1-23 (all partially)

As for invention 4, wherein said at least one of said NS5 mutations is amino acid 2199 being Thr / nucleotide 6936 of SeqIdNo.2 being adenine / nucleotide 5320 of SeqIdNo.3 being adenine.

10. claims: 1-23 (all partially)

As for invention 4, wherein said at least one of said NS5 mutations is amino acid 2204 being Arg / nucleotide 6953 of SeqIdNo.2 being adenine or guanine / nucleotide 5337 of SeqIdNo.3 being adenine

11. claims: 1, 9, 15-19 (all partially)

A nucleic acid molecule comprising an altered EMCV IRES region wherein insertion of an extra adenosine at nucleotide 1736 of SeqIdNo.3 has occurred.

12. claims: 24-39 (all totally)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Method of making an HCV replicon enhanced cell comprising the steps of introducing and maintaining a HCV replicon in a cell, and curing said cell of asaid HCV replicon to produce said replicon enhanced cell; HCV replicon enhanced-cell obtained by said method; Method of measuring the ability of a compound to affect HCV activity using said cell.
